IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellants:	Stephanie Aquin, Oliver P. Peoples, and Kristi D. Snell		
Serial No.:	09/991,152	Art Unit:	1638
Filed:	November 16, 2001	Examiner:	McElwain, Elizabeth F.
For:	*PRODUCTION OF MEDIUM CHAIN LENGTH POLYHYDROXYALKANOATES FROM FATTY ACID BIOSYNTHESIS*		

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUBSTITUTE APPEAL BRIEF

Sir:

Responsive to the Notice of Non-compliant Appeal Brief mailed on October 2, 2008, and to the Advisory Action mailed on September 11, 2008, please substitute this appeal brief for the appeal brief filed on September 10, 2008. This is an appeal from the final rejection of claims 1, 3-13, 15-20, 22-26, and 29-30 in the Office Action mailed April 10, 2008, in the above-identified patent application. A Notice of Appeal and the fee for filing a notice of appeal was previously filed on August 28, 2006. The Commissioner was authorized to charge the fee for filing an Appeal Brief for a large entity, to Deposit Account No. 50-3129, in the Appeal Brief filed on December 28, 2006.

The Examiner's attention is respectfully drawn to MPEP § 1205.02 9 *>(iii)< which states "*Status of Claims.* A statement of the status of all the claims in the application, or patent under reexamination, i.e., for each claim in the case, appellant must state whether it is cancelled,

allowed >or confirmed<, rejected, >withdrawn, objected to,< etc. Each claim on appeal must be identified". Thus, the Status of Claims in (3) below is in accordance with MPEP MPEP § 1205.02 9 *>(iii)<.

It is believed that no fee is required with this submission. However, should a fee be required, the Commissioner is hereby authorized to charge the fee to Deposit Account No. 50-3129.

(1) REAL PARTY IN INTEREST

The real party in interest of this application is Metabolix, Inc., the assignee of record.

(2) RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to the appellant, the undersigned, or appellant's assignee which directly affects, which would be directly affected by, or which would have a bearing on the Board's decision in this appeal.

(3) STATUS OF CLAIMS

Claims 1, 3-13, 15-20, 22-26, 29, and 30 are pending and on appeal. Claims 2, 14, 21, 27 and 28 have been cancelled. Claim 6 is objected to for being a duplicate of claim 5. The text of each claim on appeal, as pending, is set forth in an Appendix to this Appeal Brief.

(4) STATUS OF AMENDMENTS

Claims 1, 3-13, 15-20, 22-26, 29, and 30 were last amended in the Amendment and Response filed on March 20, 2006. Claims 27 and 28 were cancelled in the Response to Restriction Requirement filed on October 21, 2004. Claims 2, 14, and 21 were cancelled, and new claim 30 added, in the Amendment and Response filed on November 30, 2005.

(5) SUMMARY OF THE CLAIMED SUBJECT MATTER

Independent claim 1 defines a genetically engineered bacteria or plants producing polyhydroxyalkanoate (PHA), the improvement comprising

providing the organism with a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase and one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase so that medium chain length PHA accumulates through the fatty acid biosynthesis pathway (page 3, lines 20-23).

Independent claim 13 defines a method of engineering a PHA biosynthetic pathway in a transgenic organism selected from the group consisting of bacteria and plants which produce polyhydroxyalkanoate (PHA),

the improvement comprising providing the organism with one or more constructs comprising a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase and one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase so that medium chain length PHA accumulates through the fatty acid biosynthesis pathway (page 8, lines 10-17 and page 9, lines 1-6 and page 10, lines 11-26).

Independent claim 20 defines a method of making medium chain length PHA comprising growing a transgenic bacteria or plant producing PHA and expressing a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase and expressing one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase, with substrates for fatty acid biosynthesis (pages 8-10).

The organism expresses a transgene *alkK* encoding an acyl-CoA synthetase as defined by claim 4 (page 9, lines 1-6). The acyl-CoA synthesis as defined by claims 3, 15 and 22, is 3-hydroxyacyl-CoA synthetase, and by claims 5 and 6, a heterologous 3-hydroxyacyl-CoA synthetase (page 4, lines 13-18; page 7, lines 3-19), and with enhanced expression (claim 7). The organism can be a plant cell, tissue of whole plant (claims 8, 17, 18, and 19) or bacteria (claims 10 and 19) expressing a 3-hydroxyacyl-ACP thioesterase, medium chain length PHA synthase, and/or medium chain length 3-hydroxy fatty acid acyl CoA synthase (claim 8 and 10), or a 3-hydroxyacyl-ACP thioesterase, medium chain length PHA synthase, and/or medium chain length 3-hydroxy fatty acid acyl CoA synthase (claims 18 and 19) and 16, defining the method of

claim 15, and 23, 24, and 25, defining the method of claim 20, wherein the organism further expresses a PHA synthase (page 10, lines 21-26). The plant or plant cell can express marker genes, as defined by claim 9. The transgene can be targeted to a tissue or organelle such as seeds, leaf, plastids, and peroxisomes, as defined by claim 11 (page 11, line 13 until page 14, line 4) Claim 12 is where the organism is *E. coli* and PHA accumulates in the bacteria.

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues present on appeal are:

(i) whether claims 1, 3-9, 11, and 13-26 are anticipated under 35 U.S.C. §102(a) by Poirier, et al., *Plant Physiol.*, 121:1359-66 (1999) ("Poirier").

(ii) whether claims 1, 3-13, 15-20, 22-26, 29, and 30 are obvious under 35 U.S.C. §103(a) over Poirier, in view of U.S. Patent No. 5,750,848 to Kruger, et al., ("Kruger").

(iii) whether claims 1, 3-13, 15-20, 22-26, 29 and 30 are obvious under 35 U.S.C. § 103(a) in view of U.S. Patent No. 5,750,848 to Kruger, et al., ("Kruger").

(7) ARGUMENTS

(a) The Claims

Polyhydroxyalkanoates (PHAs) are natural, thermoplastic polyesters and can be processed by traditional polymer techniques for use in an enormous variety of applications, including consumer packaging, disposable diaper linings and garbage bags, food and medical products. Several factors are critical for economic biological production of PHAs, including substrate costs, fermentation time, and efficiency of downstream processing.

The enzymes in the PHA biosynthetic pathway occurring naturally in *A. eutrophus* and certain other bacteria have been elucidated. The final step in making the polymer utilizes a PHA synthase, encoded by phaC, that catalyzes the polymerization of intermediates to PHA. PHA

synthases have different substrate specificities with respect to carbon chain length, however, PHA synthases are known to utilize 3-hydroxy-acyl CoA intermediates as substrates.

Appellants have discovered a novel way to produce medium chain length PHAs in organisms that do not naturally produce them, via the fatty acid biosynthetic route, using an acyl-ACP-CoA transferase, encoded by the phaG gene. Attempts in the prior art to produce medium chain length PHAs by transforming *E. coli* with a gene encoding acyl-CoA transferase and PHA synthetase have been unsuccessful. Appellants discovered that acyl-CoA transferase under the conditions described in the prior art was functioning as a thioesterase and so was not directly providing the 3-hydroxy fatty acid substrates required by the PHA synthase. Appellants show the successful production of medium chain length PHAs by transforming organisms to express 3-hydroxyacyl-ACP thioesterase and one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase so that medium chain length PHA accumulates through the fatty acid biosynthesis pathway. None of the prior art recognized the need to provide the acyl-ACP-CoA transferase in combination with an acyl-CoA synthetase or acyl CoA transferase in order to produce PHAs. One of skill in the art could neither have arrived at, nor predicted the successful manufacture and use of, the claimed subject matter from the prior art.

(b) Rejection of claims 1, 3-9, 11, and 13-26 under 35 U.S.C. §102(a)

III. ARGUMENTS

(a) The Claimed Invention

None of the cited references either anticipate or make obvious the claimed subject matter. This is because the claims and each of the references utilize different metabolic pathways. Due

to the complexity of the metabolic pathways involved in the specification and the two cited references, Apellants feel that an explanation of these pathways may be helpful.

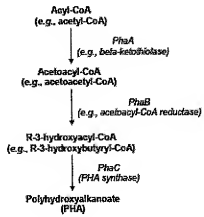
The Metabolic Pathways

The enzymes in the polyhydroxyalkanoate (PHA) biosynthetic pathway occurring naturally in *R. eutropha* and Pseudomonads are described on page 1, line 22 to page 2, line 4 of the present application. Specifically, the native pathway in *R. eutropha* typically involves:

- the conversion of acyl-CoA to acetoacyl-CoA by the action of a beta-ketothiolase enzyme (such as the enzyme encoded by the gene *phaA*), then
- the conversion of acetoacyl-CoA to a R-3-hydroxyacyl-CoA by the action of an acetoacyl-CoA reductase (such as the enzyme encoded by the gene *phaB*), then
- production of the PHA polymer utilizing a PHA synthase enzyme (such as the enzyme encoded by the gene *phaC*), which catalyzes the polymerization of the intermediate R-3-hydroxyacid-CoA to form PHA.

This is shown as follows:

(Diagram A)



In this native pathway, the precise monomer composition of the PHA is dictated predominantly by the specific identity of the R-3-hydroxyacyl-CoA that is provided by the metabolic pathway in the organism (for example, in the above scheme, the PHA would be poly-(3-hydroxybutyrate). PHA synthases are known to utilize R-3-hydroxyacyl CoA intermediates as substrates, but have different substrate specificities with respect to carbon chain length of the R-3-hydroxyacyl CoA.

The fatty acid biosynthetic pathway is capable of producing R-3-hydroxyacyl-*ACP* molecules ("ACP" is acyl carrier protein) as intermediates. However, the enzyme PHA synthase (*phaC*) cannot use R-3-hydroxyacyl-ACP as a substrate to make PHA. PHA synthase can only utilise R-hydroxyacyl-*CoA* ("CoA" is Coenzyme A) as a substrate.

Thus, the ACP intermediates of the fatty acid biosynthesis pathway could be made available for medium chain length PHA production, *if* the pathway could be engineered to include one or more metabolic steps that swap ACP for CoA on medium chain length R-3-hydroxyacyl-ACP molecules. The ACP intermediates must be converted to the CoA form in order to be acceptable substrates for PHA synthases.

The present application discloses a new way to produce medium chain length PHAs in organisms that do not naturally produce them, by tapping into the fatty acid biosynthetic route to obtain R-3-hydroxyacyl-CoA substrates for incorporation into PHA polymers. In other words, the specification discloses a new system and method of providing R-3-hydroxyacyl-CoA substrate that is independent of the naturally occurring *phaA*- and *phaB*-encoded metabolic pathway, and which leads to the production of medium chain length PHAs.

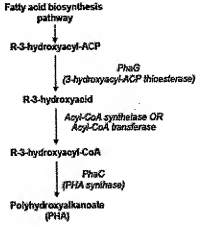
As disclosed in the specification, this was accomplished by using organisms selected and engineered to express:

(i) an enzyme with 3-hydroxyacyl-ACP thioesterase activity, such as that encoded by the *PhaG* gene, to convert R-3-hydroxyacyl-ACP into free R-3-hydroxyacid, and

(ii) an enzyme that has either an acyl-CoA synthetase OR an acyl-CoA transferase, either of which has activity to convert free R-3-hydroxyacid to R-3-hydroxyacyl-CoA.

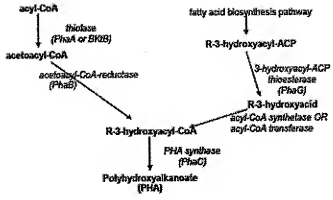
The R-3-hydroxyacyl-CoA can then be used as a substrate by a PHA synthase enzyme to produce PHA polymer. This can be represented in the following diagram as:

(Diagram B)



In effect, the pathway disclosed in the specification (shown below on right) intersects with the naturally-occurring *R. eutropha* pathway (shown on left):

(Diagram C)



As shown above, these two pathways use different initial substrates, and different pathways, to make PHA. Accordingly, the organisms must express different enzymes to utilize the pathway on the right, than are required for the pathway on the left. An organism that utilizes the pathway on the left would not inherently also be capable of possessing or being able to utilize the pathway on the right.

The enzyme *PhaG* was previously suggested to be useful in linking fatty acid biosynthesis to PHA production (see specification, page 2, line 24 to age 3, line 19), but only on the basis that it acted as an acyl-ACP CoA transferase. In other words, the prior art (including the Kruger reference cited by the examiner) suggested that *PhaG* was capable of directly converting R-3-hydroxyacyl-ACP into R-3-hydroxyacyl-CoA in a single step. However, as discussed at page 3, lines 8-14 of the present application, previous attempts to produce medium chain length PHAs by transforming *E. coli* with a gene encoding acyl-ACP-CoA transferase and medium chain length PHA synthetase were unsuccessful.

As disclosed in the present specification at page 3, line 27 to page 4, line 1, *PhaG* was found to found to function as a thioesterase rather than as an acyl-ACP-CoA transferase and so was not directly providing the 3-hydroxyacyl-CoA substrates required by the PHA synthase.

In other words, Apellants have shown that the *PhaG* enzyme alone is not capable of providing R-3-hydroxyacyl-CoA for the production of PHA from the fatty acid biosynthesis intermediate R-3-hydroxyacyl-ACP. Rather, an additional enzymatic step is required, that is, the provision of an acyl-CoA synthetase or acyl-CoA transferase enzyme that converts free R-3-hydroxyacid into R-3-hydroxyacyl-CoA.

The present specification discloses that the successful production of medium chain length PHAs from intermediates of the fatty acid biosynthetic pathway can be achieved by transforming organisms to express an enzyme having 3-hydroxyacyl-ACP thioesterase activity (e.g., *PhaG*) and one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase, so that medium chain length PHA accumulates through the fatty acid biosynthesis pathway. *PhaG* alone cannot produce PHA from R-3-hydroxyacyl-ACP.

None of the cited art recognized the need to provide *PhaG* in combination with an acyl-CoA synthetase or acyl CoA transferase in order to produce PHAs, because there was no previous recognition that *PhaG* functions as a thioesterase.

(b) Rejection of claims 1, 3-9, 11 and 13-26 under 35 U.S.C. §102(a)

Claims 1, 3-9, 11 and 13-26 were rejected under 35 U.S.C. § 102(a) as anticipated by Poirier *et al. Plant Physiology* 121:1359-1366, 1999; "Poirier".

Legal Standard

For a rejection of claims to be properly founded under 35 U.S.C. § 102, it must be established that a prior art reference discloses each and every element of the claims. *Hybritech*

Inc. v Monoclonal Antibodies Inc., 231 U.S.P.Q. 81 (Fed. Cir. 1986); *Scripps Clinic & Research Found v. Genentech Inc.*, 18 U.S.P.Q.2d 1001 (Fed. Cir. 1991).

The Federal Circuit held in *Scripps*, 18 USPQ2d at 1010:

Invalidity for anticipation requires that all of the elements and limitations of the claim are found within a single prior art reference. *There must be no difference* between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention. (Emphasis added)

A reference that fails to disclose even one limitation will not be found to anticipate, even if the missing limitation could be discoverable through further experimentation. As the Federal Circuit held in *Scripps, Id.*:

[A] finding of anticipation requires that all aspects of the claimed invention were already described in a single reference: a finding that is not supportable if it is necessary to prove facts beyond those disclosed in the reference in order to meet the claim limitations. The role of extrinsic evidence is to educate the decision-maker to what the reference meant to persons of ordinary skill in the field of the invention, not to fill in the gaps in the reference.

"A claim limitation is inherent in the prior art if it is necessarily present in the prior art, not merely probably or possibly present." *Akamai Technologies, Inc. v. Cable & Wireless Internet Services, Inc.*, 344 F.3d 1186, 1192, 68 USPQ2d 1186 (Fed. Cir. 2003).

Analysis

Poirier discloses the production of medium chain-length polyhydroxyalkanoates from intermediates of beta-oxidation of fatty acids, in order to study fatty acid degradation in developing seeds of *Arabidopsis* (Abstract). The authors created several constructs containing the *PhaC1* gene (which encodes a PHA synthase enzyme), under the control of several different

promoters. One construct was also made which included the PHA synthase gene and the *FatB3* gene, which encodes a medium chain length acyl-ACP-thioesterase.

The Poirier reference produces medium chain-length polyhydroxyalkanoates from intermediates from **fatty acid beta-oxidation.** Fatty acid beta-oxidation degrades fatty acids.

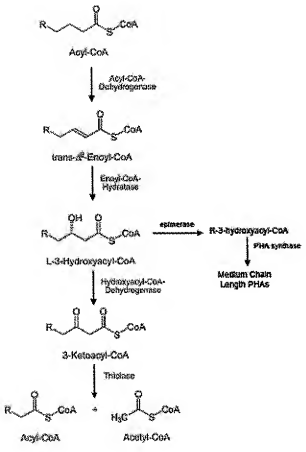
The present application produces medium chain-length polyhydroxyalkanoates from intermediates from **fatty acid biosynthesis.** Fatty acid biosynthesis builds and elongates fatty acid chains.

The two are very different pathways, and are not mirror-image reversals of each other. They involve different enzymes and steps.

The beta-oxidation fatty acid degradation cycle acts on long-chain fatty acids, and through four steps, removes two carbons in the form of an acetyl-CoA. The fatty acid, now two carbons shorter, goes through the cycle again, which produces another two-carbon acetyl-CoA and a fatty acid chain reduced by those two carbons. The cycle is repeated until the fatty acid is completely degraded to acetyl-CoA. This is shown in Diagram D below.

During the cycle, S-3-hydroxyacyl-CoA is produced as an intermediate. This molecule can be diverted to make medium chain-length polyhydroxyalkanoate *if* an epimerase is provided to convert the S-3-hydroxyacyl-CoA to R-3-hydroxyacyl-CoA. PHA synthase can only act upon R-3-hydroxyacyl-CoA.

(Diagram D)



The claimed subject matter does not involve the beta-oxidation pathway. Instead, it uses a product of the fatty acid biosynthesis pathway as its raw material.

In contrast to the beta-oxidation pathway, fatty acid biosynthesis builds and elongates fatty acid chains. During this elongation cycle, R-3-hydroxyacyl-ACP is made. PHA synthase cannot act on this molecule, and so the R-3-hydroxyacyl-*ACP* must be converted to R-3-hydroxyacyl-*CoA*. This is accomplished by a two-step process (see Diagram B, above). First, a thioesterase, *e.g.*, R-3-hydroxyacyl-ACP thioesterase, converts the R-3-hydroxyacyl-ACP to an R-3 hydroxyacid. This molecule must then be converted to R-3-hydroxyacyl-CoA by either a

synthetase (*e.g.*, acyl-CoA-synthetase) or a transferase (*e.g.*, acyl-CoA-transferase). Once the conversion to R-3-hydroxyacyl-CoA has occurred, a PHA synthase can act on this substrate, and convert it to PHA.

The Examiner's states that Apellants "assert" that the production of medium chain length PHA from intermediates of fatty acid beta-oxidation instead of from intermediates of fatty acid biosynthesis are very different pathways involving different enzymes and steps. This is not an assertion but fact. Evidence is presented in the application as filed and in the prior art cited by both parties in support of these statements. The Examiner has provided no evidence to the contrary. Therefore any analysis of the claims under 102 or under 103 from proceed from this basis. In fact there are differences in (1) the enzymes, (2) the substrates which are provided to the organisms, and (3) the substrates which are produced by the enzymes during the process. These differences are both explicit and inherent in the disclosed and claimed subject matter as well as the prior art.

Porier does not disclose each and every element of the claims.

The claims define a genetically engineered organism selected from the group consisting of bacteria and plants producing polyhydroxyalkanoate (PHA), the improvement comprising

providing the organism with

(1) a **transgene** encoding an enzyme having **the catalytic activity of 3-hydroxyacyl-ACP thioesterase** and

(2) **one or more transgenes** encoding enzymes having the catalytic activity of **acyl-CoA synthetase or acyl CoA transferase**

so that medium chain length accumulates through the fatty acid biosynthesis pathway.

Porier discloses plant transformation for PHA synthesis using a Pha*C1* synthase gene from *Pseudomonas aeruginosa* and the *Cuphea lanceolata* FATB3 gene, encoding a medium-chain length acyl-ACP thioesterase (Poirier, page 1360, right col.). The genetically engineered organism disclosed in Poirier is different from the organisms defined by the claims in at least two respects:

(i) **the plants in Poirier do not contain a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase** and

(ii) **the plants in Poirier do not contain a transgene encoding enzymes having the catalytic activity of an acyl-CoA synthetase or acyl CoA transferase.**

The Examiner stated that the claimed genetically engineered organism comprising coding sequences for a PHA synthase and a medium chain length acyl-ACP-thioesterase that uses an intermediate from *the fatty acid biosynthetic pathway* would not be distinguishable from a genetically engineered organism comprising coding sequences for a PHA synthase coding sequence and a medium chain length acyl-ACP-thioesterase that uses an intermediate from *the fatty acid degradation pathway* produced by beta oxidation. This is not correct. **The Examiner has ignored the limitation that the claimed organisms also have a transgene encoding enzymes with the catalytic activity of acyl-CoA synthetase or acyl CoA transferase.**

Furthermore, the claimed organisms have a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase. The claimed organisms do not merely comprise a medium chain length acyl-ACP-thioesterase as stated by the Examiner, the thioesterase has 3-hydroxyacyl-ACPthioesterase activity. The distinction lies in the fact that the medium chain length acyl-ACP thioesterase employed in Porier is an enzyme that takes part in terminating fatty acid biosynthesis, therefore, releasing free fatty acids from ACP [see discussion

of Poirier in Yves Poirier, *Progress Lipid Res.* 41:131-155 (2002) ("Yves"; see especially page 150, 3.3.3 under "Studies on Futile Cycling of Fatty Acids)]. According to Yves, previous studies using lauroyl-ACP thioesterase (utilized in Poirier), indicated that expression of a thioesterase might be a way of increasing the carbon flux toward β-oxidation and peroxisomal PHA synthesis (see Yves, Figure 6). Figure 6 in Yves shows the release of fatty acids from the fatty acid synthesis pathway, which are then channeled through the peroxisomal β-oxidation pathway to eventually obtain the R-3-hydroxy-acyl-CoA substrate for PHA synthase. Yves further states that the relationship between fatty acid futile cycling and peroxisomal PHA synthesis extends to developing seed, citing to the studies by Poirier (see Yves, page 150, 3.3.3, last para.). Thus, lauroyl-ACP thioesterase (medium chain length acyl-ACP thioesterase) employed in Poirier releases free fatty acids which undergo additional reactions (postulated in Yves figure 6) to produce 3-hydroxyacyl-CoA.

In contrast, the claimed organisms contain a transgene encoding an enzyme capable of directly converting 3-hydroxy-acyl-ACP to 3-hydroxyacyl-CoA. The Examiner has provided no evidence that lauroyl-ACP thioesterase possesses this activity. Even if they did, the organisms are still different because the claims require that the organism additionally comprise a **transgene** encoding an acyl-CoA transferase/synthetase activity. This is not disclosed in Poirier.

The Examiner also stated (office action, page 3, 2nd para.) that the acyl-CoA synthetase that polymerizes the 3-hydroxyacyl-CoA intermediates would be a hydroxyacyl-CoA synthetase, and since Apellants have used PHA synthase and PHA synthetase interchangeably, the particular name of the transgene such as *alkK*, does not distinguish it from others having the same activity. Apellants are unclear as to the Examiner's point here, since acyl-CoA synthetase does not polymerize 3-hydroxyacyl-CoA intermediates; PHA synthase (also known in the art as PHA

synthetase or PHA polymerase) does. Acyl-CoA synthetase (such as alkK) catalyzes the transfer of the CoA moiety from CoA-SH to acyl groups. Poirier does not disclose transforming an organism with acyl-CoA synthetase or acyl CoA transferase.

Clams 3 and 4- 6 are novel over Poirier

In addition to the arguments above with respect to claim 1, Poirier does not disclose providing an organism with a transgene encoding any acyl-CoA synthetase, even less so a 3-hydroxyacyl-CoA synthetase or the *alk* gene. Therefore, claims 3 and 4-6 are novel over Poirier.

Claims 13 and 15 are novel over Poirier

Poirier does not disclose providing a construct comprising a transgene encoding the catalytic activity of acyl CoA-synthetase or acyl CoA transferase as required by the claims. Therefore, claims 13 and 15 are novel over Poirier.

Claims 16-18 are novel over Poirier

Claims 16-18 depend from claim 13 and additionally require the construct further comprise a transgene encoding a PHA synthase. Poirier does not disclose a method of providing an organism with gene constructs having three enzyme activities. Therefore, claims 16-18 are novel over Poirier.

Claims 20 and 23-25 are novel over Poirier

Poirier does not disclose a method of making medium chain length PHA as claimed, that requires growing an organism producing PHA and expressing a transgene encoding an enzyme having the catalytic activity of 3-hydroxyl-ACP thioesterase and expressing one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase. Therefore, claims 20, and 23-25 are novel over Poirier.

Claims 22 and 24 are novel over Poirier

Claims 22 and 24 depend on claim 20 and specifically define the acyl-CoA synthetase as 3-hydroxyacyl-CoA synthetase. Poirier does not disclose a method for making medium chain length PHA that involves providing any acyl-CoA synthetase, even less so a 3-hydroxyacyl-CoA synthetase. Therefore, claims 22 and 24 are novel over Poirier.

Claim 26 is novel over Poirier

Poirier does not disclose a method of making PHA in bacteria as admitted by the Examiner (page 4 of Examiner's Answer mailed on 10/18/07). Therefore, claim 26 is novel over Poirier.

(c) Rejection of Claims 1, 3-13, 15-20, 22-26, 29 and 30 as obvious under 35 U.S.C. §103(a) over Poirier in view of U.S. Patent No. 5,750,848 to Kruger

Rejection of Claims 1, 3-13, 15-20, 22-26, 29 and 30 as obvious under 35 U.S.C. §103(a) over U.S. Patent No. 5,750,848 to "Kruger".

Legal Standard

The starting point for any such analysis must be the Supreme Court's decision in *KSR International Co. v. Teleflex, Inc,* 127 S. Ct 1739 (2007), which refocuses the determination of whether a claimed invention is obvious back to the process the Court had defined in *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1, 17-18 (1966). The Court held that the obviousness determination should address four factors, all of which must be considered, though not in any prescribed order: (1) the scope and content of the prior art; (2) the level of ordinary skill in the art; (3) the differences between the claimed invention and the prior art; and (4) any secondary considerations suggesting nonobviousness, such as commercial success, failure of others, and long felt but unmet need. *Id.* The Court cautioned that the fact finder should be careful about

reading the teachings of the invention at issue into the prior art, to avoid applying inappropriate hindsight, *ex post* reasoning. *Id.* at 36.

In the chemical arts, where compounds are so similar as to create an expectation that the claimed new compound would have similar properties as the prior art compounds, the Federal Circuit also has upheld a finding that the claimed invention is not patentable. *Aventis Pharma Deutschland GmbH v. Lupin, Ltd.*, 499 F.3d 1293, 1301 (Fed. Cir. 2007). However, when the prior art disclosed a broad selection of compounds that might have been potential candidates for further investigation, the lack of sufficient guidance and predictability to select the compound at issue supported a finding of nonobviousness. *Takeda Chem. Indus. Ltd. v. Alphapharm Pty., Ltd.*, 492 F.3d 1350, 1359-60 (Fed. Cir. 2007), *petition for cert. filed*, 76 U.S.L.W. 3374 (U.S. Dec. 20, 2007) (No. 07-838); *see also In re Sullivan*, 498 F.3d 1345 (Fed. Cir. 2007) (remanding to the Board, noting that despite close similarity of the claimed invention and prior art, rebuttal evidence to which the Board gave inadequate consideration showed unexpected results, a teaching away from appellant's invention and a long felt but unmet need).

Even where the prior art suggests or motivates an inventor to develop the composition or process at issue, the Federal Circuit continues to recognize that there is a critical question under 35 U.S.C. § 103 as to whether the combined teachings of the prior art "would have given rise to a reasonable expectation of success" in achieving what is claimed. *PharmaStem Therapeutics, Inc. v. ViaCell, Inc.*, 491 F.3d 1342, 1360 (Fed. Cir. 2007), *petition for cert. filed*, 76 U.S.L.W. 3393 (U.S. Jan. 2, 2008) (No. 07-888).

Analysis

The scope and contents of the prior art

Poirier

Poirier is discussed above.

Kruger

Kruger discloses a genomic fragment harboring the *PhaG* gene, cloned by complementation of *Pseudomonas putida* mutants defective in PHA synthesis via *de novo* fatty acid biosynthesis. According to Kruger, the *PhaG* gene is useful for the production of PHAs in bacteria and plants (Kruger, abstract).

Kruger proposes a myriad of possible activities for the isolated *PhaG* (discussed below);

Col. 3, lines 16-24 suggests, at best, a CoA-ACP acyltransferase activity for *PhaG*, which directly converts acyl-ACP molecules to acyl-CoA molecules.

Example 10 further notes that transforming organisms with transgenes for just the *PhaG* enzyme and PHA synthase would be sufficient for the production of PHAs. This teaches the reader that the *PhaG* enzyme is capable of directly converting acyl-ACP intermediates to acyl-CoA substrates for PHA synthase.

Kruger speculates on a plethora of possible other activities for *PhaG*. The reference states (see, *e.g.*, col. 5, lines 62-64 and col. 6, lines 2-8) that:

(i) *PhaG* may be an R-3-hydroxyacyl CoA-ACP acyl transferase, catalyzing the conversion of R-3-hydroxyacyl-ACP to R-3-hydroxyacyl CoA;

(ii) *PhaG* may be a CoA-ACP acyltransferase with an acyl group specificity other than the 3-hydroxy functionality. Referring to Figure 1 in Kruger therefore, *PhaG* could be

an acyl-ACP CoA transferase

a 3-Ketoacyl-ACP CoA transferase or,

an enoyl-ACP CoA transferase

(iii) *PhaG* may have activity associated with a specific thioesterase (active on any of the acyl-ACP forms in Figure 1), in which case the enzyme encoded by *PhaG* additionally requires a ligase. Referring to Figure 1, *PhaG* could also be

an acyl-ACP thioesterase,

a 3-hydroxyacyl-ACP thioesterase,

a 3-ketoacyl-ACP thioesterase or,

an enoyl-ACP thioesterase

(iv) *PhaG* may have activity associated with a ligase, in which case *PhaG* additionally requires a thioesterase. Referring to Figure 1, therefore, *PhaG* could be

a ligase specific for the fatty acid released from acyl-ACP,

a ligase specific for the fatty acid released from 3-hydroxyacyl-ACP,

a ligase specific for the fatty acid released from 3-ketoacyl-ACP,

a ligase specific for the fatty acid released from enoyl-ACP.

(v) *PhaG* may be a protein that stabilizes a catalytic protein complex which catalyzes

the acyl group transfer reaction or,

thioesterase or

ligase activity; or

(vi) *PhaG* may be a protein that regulates a catalytic protein complex which catalyzes,

the acyl group transfer reaction or,

thioesterase or,

ligase activity.

Referring to (i) to (iv) above, *PhaG* could have any of 12 different enzymatic activities. Of note is the fact that, for each of the 12 possible enzymatic activities, the set of additional enzymes required to arrive at PHA are different.

Referring to (v) and (vi) above, PhaG may not be an enzyme at all. It may just stabilize any of the four acyltransferases shown in Figure 1, stabilize a thioesterase or ligase; thus PhaG may stabilize any of 16 different enzymes. PhaG may also be a regulator, regulating any of the 4 acyl-acyltransferases, thioesterases or ligases; thus, PhaG may regulate any of 16 different enzymes.

As one of ordinary skill in the art knows from a review of any Molecular Biology Text book, the regulation of a protein can occur at the transcriptional level, translational level or post translational level. Regulation of protein activity or stabilization of a protein for example, can be accomplished by protein-protein interactions (in which case *PhaG* does not need to be an enzyme) or by reversible or irreversible posttranslational enzymatic modifications. See summary of different post translational modifications possible retrieved from **http://en.wikepedia.org/wiki/Post-translational_modification** on 7/10/2008. Of note is the fact that there are numerous ways by which a protein can regulate/stabilize another protein as demonstrated by the summary for possibilities of post translational modification alone. Thus, assigning a regulatory/stabilization role to *PhaG when the authors of the papers make explicit that they do not know what the enzyme or other molecule represented by PhaG is* presents an infinite number of possible roles for PhaG with respect to regulating or stabilizing the 16 possible enzymes discussed above.

However, when the reference is considered as a whole it is clear (particularly from col. 3, lines 16-24 and Example 10) that the Kruger authors considered *PhaG* to be a 3-hydroxy acyl-ACP-CoA transferase.

Ascertaining the differences between the prior art and the claims

Kruger

Kruger considered alone, does not disclose all of the elements of the claims.

Although Kruger discloses the expression of *PhaG*,

(i) it does not recognize that *PhaG* acts as a 3-hydroxyacyl-ACP thioesterase;

(ii) **it does not disclose or suggest that a 3-hydroxyacyl-ACP thioesterase must be co-expressed with one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase**; and

(iii) it does not disclose that the claimed system can be used to allow medium chain length PHAs accumulate.

The Examiner is incorrect (office action page 8, 2nd para.) in stating that Kruger suggests that there may be both acyltransferase activity and thioesterase activity associated with *PhaG*. **Kruger does not disclose or suggest dual activity for *PhaG*.**

The Examiner stated that the *PhaG* sequence that Apellants use is the same as that taught by Kruger, which would inherently have 3-hydroxyacyl-ACP thioesterase activity (office action page 9, 1st para.). However, it is the recognition that *PhaG* has thioesterase activity by the Apellants that necessitates the provision of the additional transgenes which provide the necessary enzyme activity to make polymer via the claimed pathway. The claimed organisms comprise transgenes encoding **3-hydroxyacyl-ACP thioesterase and one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase.**

The Examiner also stated that Kruger discloses that *PhaG* can be used in conjunction with other PHA biosynthetic enzymes, and the evidence for non-obviousness should be commensurate with the scope of the claims. It appears as though the Examiner is interpreting "PHA biosynthetic enzyme" as used in Kruger to mean any enzyme (such as the enzymes disclosed in Figure 1) of Kruger that can employed in the making of PHA. This is incorrect. Such reasoning would classify the medium chain acyl-thioesterase (a terminal fatty acid biosynthetic enzyme) used in Poirier to increase the supply of free fatty acids for PHA biosynthesis, a "PHA biosynthetic enzyme". One of ordinary skill in the art would not arrive at this conclusion. Furthermore, attention is respectfully drawn to Figure 1 in Kruger, under the heading "PHA Biosynthesis" and the pathway disclosed below that heading which includes the enzymes β-ketothiolase, 3-ketoacyl-CoA reductase and PHA synthase. Without a definition of PHA biosynthetic enzymes in Kruger, it is clear that this would be the enzymes listed under PHA Biosynthesis.

One of ordinary skill in the art would not interpret the phrase "PHA biosynthetic enzymes" to be in reference acyl-CoA transferase/synthetase. The Examiner maintained that Apellants did not provide a definition of PHA biosynthetic enzymes in the specification that would exclude those taught by Kruger. Kruger does not teach acyl-CoA synthetase/transferase as a "PHA biosynthetic enzyme". Apellants provided evidence as to which enzymes one of ordinary skill in the art would understand are PHA biosynthetic enzymes (*See* Madison and Huisman, *Microbiol and Mol. Biol. Rev.*, 63(1):21-53 (1999) ("Madison" attached to the Appeal Brief filed on December 28, 2006)). Apellants additionally point to Kruger (paragraph bridging col. 23 and 24.) where Kruger states "optimal PHA synthesis via de novo fatty acid biosynthesis in bacteria and plants comprises at least two genes: PHA synthase (phaC) and phaG (disclosed

herein). Methods for incorporating PHA synthesis and other PHA genes (phaA-β-ketothiolase) and phaB (D-reductase) genes into transformation and expression vector constructs....are well known in the art". Clearly, similar to the disclosure in Madison, Kruger is referring to phaA, phaB and phaC when the phrase "PHA biosynthetic genes" is used. The Examiner has provided no evidence for the assertion that acyl-CoA transferase/synthetase would be considered "A PHA biosynthetic enzyme". Even if the Examiner did, one would still have to select the role of thioesterase from the myriad of possible roles suggested for in Kruger for *PhaG* (discussed above) in order to additionally select an acyl-CoA transferase/synthetase activity and arrive at the claims.

Porier in Combination with Kruger

As discussed above in response to the 102(a) rejection, Poirier does not disclose the claimed organisms; Kruger does not make up for this deficiency. The Examiner depended on Kruger for disclosing genetically engineered bacteria. However, a combination of Kruger and Poirier does not disclose a bacteria or plant comprising transgenes encoding enzymes having the catalytic activity of a 3-hydroxyacyl-ACP thioesterase **and** acyl-CoA synthetase or acyl CoA transferase.

The prior art considered alone or in combination, does not disclose the need to provide an acyl coenzyme A synthetase or acyl CoA transferase in combination with *PhaG* (known in the prior art as an acyl-ACP-CoA transferase – see Yves, page 146, 3.24, or Madison, page 33, left col.) organisms so that medium chain length PHA can accumulate.

Part of the Examiner's allegation of obviousness is based on the Examiner's observation that Kruger at column 3, line 46 and in Figure 1, relates thioesterase activity to *PhaG*. However, due to the myriad of infinite possibilities for the function disclosed in Kruger for *PhaG*, the

Examiner is using an improper "Obvious to try rationale", (which invites one to try each of numerous possible choices until one possibly arrived at a successful result) in support for the obviousness rejection. As stated in the MPEP § 2145(B), "The admonition that 'obvious to try' is not the standard under § 103 has been directed mainly at two kinds of error. In some cases, what would have been 'obvious to try' would have been to **vary all parameters (emphasis added)** or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful". There is no predictability from Kruger to the claimed subject matter because there were too many options, and insufficient guidance in the prior art.

This is exactly the case with respect to Kruger. For each of the twelve possible enzyme activities disclosed for *PhaG* from (i) to (iv) above, one would need to provide completely different enzyme combinations in addition to PhaG in each case, in order to arrive at PHA (*see* FIG 1 of Kruger); for example,

if *PhaG* has the enzyme activity of (i) above, only a PHA synthase is needed;

if *PhaG* is (ii), then for each of the three different acyl-group functionalities disclosed in FIG 1, either

(a) a 2,3,-enoyl-CoA D hydratase will be needed in addition to the PHA synthase or

(b) a 3-ketoacyl-CoA reductase in addition to the PHA synthase or

(c) β-ketothiolase (phaA) and a 3-ketoacyl-CoA reductase (phaB) in addition to the PHA synthase.

if *PhaG* is (iii) above, then an enzymes with ligase activity is needed in addition to a phaA, phaB and PHA synthase.

if *PhaG* is a ligase (iv), then an enzyme with thioesterase activity is needed in addition to phaA, phaB and PHA synthase.

With respect to (v) above, *PhaG* might not even have any catalytic activity in the pathway shown in Fig 1 of Kruger, but might instead regulate or stabilize an one of the enzymes in the pathway. Clearly, attributing a thioesterase activity to *PhaG* is nothing but an obvious to try rationale.

A reference must be considered as a whole in order to determine what it would convey to one of ordinary skill in the art. From the consideration of Kruger as a whole, it is clear that Kruger ascribes an (R)-3-hydroxyacyl-CoA ACP transferase activity, since Kruger discloses that *PhaG* and phaC (PHA synthase) are sufficient for PHA synthesis (*see* from col. 23, line 66, until col. 24, line 1). See also, the disclosure in Kruger at least at col. 3, lines 21-25. This is supported by the discussion of the Kruger studies in Yves (see page 146, 3.2.4). Yves (an example of one of skill in the art) discusses the studies in Kruger, stating that *PhaG* catalyzes the conversion of R-3-hydroxyacyl-ACP to R-3-hydroxyacyl-CoA, the later being the substrate for PHA synthase. See also Madison, page 33, left column. Clearly the interpretation of the studies patented in Kruger by two separate skilled artisans, supports Apellants assertion that one of ordinary skill in the art on considering Kruger as a whole would consider PhaG as an (R)-3-hydroxyacyl-CoA ACP transferase catalyzing the conversion of 3-hydroxyacyl-ACP to 3-hydroxyacyl-CoA.

There is no motivation for one of ordinary skill in the art to combine Poirier and Kruger or to modify Kruger, because Kruger teaches away from the claimed organisms

A reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the Applicant. The degree of teaching away will of course depend on the particular facts; in general, a reference will teach away if it suggests that the line of development flowing from the reference's disclosure is unlikely to be productive of the result sought by the Applicant. See *United States v. Adams*, 383 U.S. 39, 52, 148 U.S.P.Q. (BNA) 479, 484, 15 L. Ed. 2d 572, 86 S. Ct. 708 (1966) ("known disadvantages in old devices which would naturally discourage the search for new inventions may be taken into account in determining obviousness"); *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1550-51, 220 U.S.P.Q. (BNA) 303, 311 (Fed. Cir. 1983) (the totality of a reference's teachings must be considered), cert. denied, 469 U.S. 851 (1984); *In re Caldwell*, 50 C.C.P.A. 1464, 319 F.2d 254, 256, 138 U.S.P.Q. (BNA) 243, 245 (CCPA 1963) (reference teaches away if it leaves the impression that the product would not have the property sought by the Applicant).

Kruger, hypothesizes among an infinite number of possibilities, that *PhaG* could be an acyl-ACP thioesterase; however, further discussion in Kruger would lead a skilled artisan to think that the most probable activity for this protein according to Kruger would be a CoA-ACP transferase activity (*see* Kruger, column 3, lines 20-24), since Kruger in Example 10 states that at least the *PhaG* enzyme and PHA synthase would be sufficient for the production of PHAs. This implies that the *PhaG* enzyme is capable of directly converting (R)-3-hydroxyacyl-ACP intermediates to (R)-3-hydroxyacyl-CoA substrates for PHA synthase. This activity has in fact been previously demonstrated (*see* the specification at least from page 2, line 24 until page 3,

line 5). Therefore, there would be no motivation for one of ordinary skill in the art to modify the disclosure in Kruger to arrive at the claimed organisms and method. In response, the Examiner (at page 8, 3rd para.) stated that the claims do not require that the organism be transformed with an acyl-CoA transferase, the claims only require that the organism be transformed with either an acyl-CoA synthetase or an acyl-CoA transferase along with a 3-hydroxyacyl-ACP thioesterase. Apellants are unclear as to the Examiner's point and it would appear that there is confusion about the role of an acyl-ACP-CoA transferase (ascribed to *PhaG* in the prior art), and acyl-CoA transferase recited in the claims. The point here is that if *PhaG* has the ability to directly transfer a coA moiety to the 3hydroxyacyl-ACP intermediates, there would be no need for the acyl-CoA transferase activity recited in the claims; thus, one of ordinary skill in the art would not be motivated to modify the disclosure in Kruger to arrive at the claims.

The Examiner has cited a desire to produce PHA in bacteria as motivation to combine Kruger and Porier. However, a combination of Kruger and Porier does not arrive at the claimed subject matter. Poirier coexpresses a plastidial acyl-ACP thioesterase and a peroxisomal PHA synthase to increase accumulation of PHA in developing seeds-the thioesterase which functions in the terminal step in fatty acid biosynthesis, is used in Poirier to increase the flux of free fatty acids from the fatty acid biosynthetic chain into the PHA biosynthetic pathway. There is no motivation for one of ordinary skill in the art to modify the combination of Poirier and Kruger to arrive at the claims which require transgenes encoding enzymes with acyl-CoA transferase or synthetase activity, because from the disclosure in Kruger a skilled artisan would believe that there was **no need** for an acyl transferase to be engineered into the same organism expressing PhaG (PhaG is an acyl transferase according to Kruger), and would not be motivated to do so.

Clams 3 and 4-6 are non-obvious over Kruger and Kruger and Porier

In addition to the arguments above with respect to claim 1, neither Kruger nor Kruger and Porier disclose providing an organism with a transgene encoding any acyl-CoA synthetase, even less so a 3-hydroxyacyl-CoA synthetase or the *alkK* gene. Therefore, claims 3 and 4-6 are non-obvious over Kruger or Kruger and Porier.

Claims 13 and 15 are non-obvious over Kruger or Kruger and Porier

Neither Kruger nor Kruger and Porier disclose providing a construct comprising a transgene encoding the catalytic activity of acyl CoA-synthetase or acyl CoA transferase as required by the claims. Therefore, claims 13 and 15 are non-obvious over Kruger or Kruger and Porier.

Claims 16-18 are non-obvious over Kruger or Kruger and Porier

Claims 16-18 depend from claim 13 and additionally require the construct further comprise a transgene encoding a PHA synthase. Neither Kruger nor Kruger and Porier disclose a method of providing an organism with gene constructs having three enzyme activities. Therefore, claims 16-18 are non-obvious over Kruger or Kruger and Porier.

Claims 20 and 23-25 are non-obvious over Kruger or Kruger and Porier

Neither Kruger nor Kruger and Porier disclose a method of making medium chain length PHA as claimed, that requires growing an organism producing PHA and expressing a transgene encoding an enzyme having the catalytic activity of 3-hydroxyl-ACP thioesterase and expressing one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase. Therefore, claims 20, and 23-25 are non-obvious over Kruger or Kruger and Porier.

Claims 22 and 24 are non-obvious over Kruger or Kruger and Porier

Claims 22 and 24 depend on claim 20 and specifically define the acyl-CoA synthetase as 3-hydroxyacyl-CoA synthetase. Neither Kruger nor Kruger and Porier disclose a method for making medium chain length PHA that involves providing any acyl-CoA synthetase, even less so a 3-hydroxyacyl-CoA synthetase. Therefore, claims 22 and 24 are non-obvious over Kruger nor Kruger and Porier.

Claim 26 is non-obvious over Kruger or Kruger and Porier

Neither Kruger nor Kruger and Porier disclose a method of making PHA in bacteria as admitted by the Examiner (page 4 of Examiner's Answer mailed on 10/18/07). Therefore, claim 26 is non-obvious over Kruger or Kruger and Porier.

Secondary Considerations of Obviousness

Secondary considerations to be considered include commercial success, long felt but unresolved needs, failure of others, unexpected results, etc.

The results shown in Example 4 (discussed below) demonstrate success where others have failed in using *PhaG* for the production of PHA in transgenic organisms.

Yves at least at page 146 (3.2.4) discloses that co-expression of the PHA synthase and *PhaG* did not conclusively lead to PHA accumulation in plant plastids (citing to unpublished data by Mittendorf). Of note is the fact that this method of using *PhaG* is in accordance with the disclosure in Kruger i.e. PHA synthase plus *PhaG* is sufficient (see Kruger, example 10). Thus, although there is no data in Kruger showing accumulation of PHA in any organism, the studies discussed in Yves demonstrate that at least in plants engineered to express *PhaG* and PHA synthase, PHA does not accumulate. This is confirmed in Example 5 of the present application which shows the inability to obtain PHA accumulation in plant chloroplasts, using PHA synthase

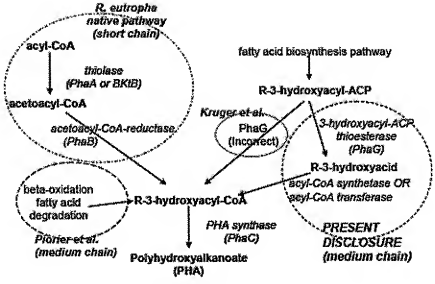
and *PhaG* alone. Similar results were obtained with *E. coli* (see Examples 2 of the present application); *E. coli* strains transfected with a plasmid containing PHA synthase and *PhaG* alone did not accumulate any PHA. However, Example 4 shows the accumulation of PHA in E. coli which was additionally transfected with the acyl-CoA synthetase activity of *alkK* (an example of the claimed organism, transformed according to the claimed method). Thus, the claims provide genetically engineered organism which express PHA and PHA production using *PhaG* where others have failed.

From the detailed analysis of the Graham factors discussed above, the Examiner has failed to establish a *prima facie* case of obviousness and claims 1, 3-13, 15-20, 22-26, 29, and 30 are not obvious over Kruger alone or in combination with Poirier.

(d) Summary and Conclusions

The four metabolic pathways (the native *R. eutropha* pathway, the Apellants' engineered pathway, the Kruger pathway, and the Poirier pathway) are highly complex and demonstrated by the following diagram, showing the four pathways together, demonstrating how they each occupy "space" that is separate from the others:

(Diagram E)



From this diagram it can be seen that all four pathways are distinct from each other. All four pathways are capable of eventually making R-3-hydroxyacyl-CoA, from which polyhydroxyalkanoate can be made. However, all four reach R-3-hydroxyacyl-CoA in completely different ways. Apellants' claims to a three-enzyme transgene system for making PHA (dashed circle in lower right) is therefore distinct from that of Kruger (a (probably incorrect) two-enzyme system; small dashed oval in middle of diagram), Poirier (which uses beta-oxidation to obtain R-3-hydroxyacyl-CoA; dashed oval in lower left), and the native *R. eutropha* system (which uses a thiolase and a reductase; large dashed oval at upper left). The claims are therefore novel over Poirier.

Kruger, alone or in combination, cannot render obvious the subject matter of Apellants' claims, because this reference fails to disclose that in addition to *PhaG*, either an acyl-CoA synthetase or an acyl-CoA transferase is required to make PHA. As disclosed by Apellants, providing *PhaG* is not enough. The Kruger reference neither supplies the missing elements of

Apellants' claims, nor provides any suggestion or guidance as to how its teachings can be modified to provide the subject matter of the present claims. Poirier fails to make up for this deficiency. Therefore the claims are non-obvious over Kruger, Porier or Kruger in combination with Kruger.

Accordingly, claims 1, 3-13, 15-20, 22-26, 29, and 30 are novel and non-obvious over Poirier, Kruger alone or Kruger in combination with Poirier.

Respectfully submitted,

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Claims Appendix: Claims On Appeal

1. A genetically engineered organism selected from the group consisting of bacteria and plants producing polyhydroxyalkanoate (PHA),

the improvement comprising providing the organism with a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase and one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase so that medium chain length PHA accumulates through the fatty acid biosynthesis pathway.

3. The organism of claim 1 wherein the acyl-CoA synthetase is 3-hydroxyacyl-CoA synthetase.

4. The organism of claim 1 comprising a transgene alkK encoding an acyl-CoA synthetase.

5. The organism of claim 1 expressing a heterologous 3-hydroxyacyl-CoA synthetase activity.

6. The organism of claim 1 expressing a heterologous 3-hydroxyacyl-CoA synthetase activity.

7. The organism of claim 1 wherein the enzyme is modified to enhance expression in the genetically engineered organism.

8. The organism of claim 1 expressing an enzyme selected from the group consisting of 3-hydroxyacyl-ACP thioesterase, medium chain length PHA synthase, and medium chain length 3-hydroxy fatty acid acyl CoA synthase, wherein the organism is a plant cell, plant tissue, or whole plant.

9. The organism of claim 8 further expressing selectable marker genes, wherein the organism is a whole plant.

10. The organism of claim 1 expressing an enzyme selected from the group consisting of 3-hydroxyacyl-ACP thioesterase, a PHA synthase that incorporates medium chain length hydroxy acids, and medium chain length 3-hydroxy fatty acid acyl CoA synthetase, wherein the organism is a bacteria.

11. The organism of claim 8 wherein expression of the transgene is targeted to a tissue or organelle selected from the group consisting of seeds, leaf, plastids, and peroxisomes.

12. The organism of claim 10 wherein the bacteria is *E. coli* and PHA accumulates within the bacteria.

13. A method of engineering a PHA biosynthetic pathway in a transgenic organism selected from the group consisting of bacteria and plants which produce polyhydroxyalkanoate (PHA),

the improvement comprising providing the organism with one or more constructs comprising a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase and one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase so that medium chain length PHA accumulates through the fatty acid biosynthesis pathway.

15. The method of claim 13 wherein the construct comprises a transgene encoding a 3-hydroxy acyl-CoA synthetase.

16. The method of claim 15 wherein the construct further comprises a transgene encoding a PHA synthase.

17. The method of claim 16 wherein the organism is a plant.

18. The method of claim 16 wherein the construct expresses an enzyme selected from the group consisting of 3-hydroxyacyl-ACP thioesterase, medium chain length PHA synthase, and medium chain length 3-hydroxy fatty acid acyl CoA synthase, wherein the organism is a plant cell, plant tissue, or whole plant.

19. The method of claim 16 wherein the construct expresses an enzyme selected from the group consisting of 3-hydroxyacyl-ACP thioesterase, a PHA synthase that incorporates medium chain length hydroxy acids, and medium chain length 3-hydroxy fatty acid acyl CoA synthetase, wherein the organism is a bacteria.

20. A method of making medium chain length PHA comprising growing a transgenic organism selected from the group consisting of bacteria and plants, the organism producing polyhydroxyalkanoate (PHA) and expressing a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase and expressing one or more transgenes encoding enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase, with substrates for fatty acid biosynthesis.

22. The method of claim 20 wherein the acyl-CoA synthetase is 3-hydroxyacyl-CoA synthetase.

23. The method of claim 20 wherein the organism further express a PHA synthase.

24. The method of claim 22 wherein the organism further express a PHA synthase.

25. The method of claim 24 wherein the organism expresses an enzyme selected from the group consisting of 3-hydroxyacyl-ACP thioesterase, medium chain length PHA synthase, and medium chain length 3-hydroxy fatty acid acyl CoA synthase, wherein the organism is a plant cell, plant tissue, or whole plant.

26. The method of claim 24 wherein the organism expresses an enzyme selected from the group consisting of 3-hydroxyacyl-ACP thioesterase, a PHA synthase that incorporates medium chain length hydroxy acids, and medium chain length 3-hydroxy fatty acid acyl CoA synthetase, wherein the organism is a bacteria.

29. The organism of claim 10 wherein the bacteria is *E. coli*, the bacteria expresses 3-hydroxyacyl-ACP thioesterase and wherein 3-hydroxy acids are secreted into the culture medium.

30. The method of claim 13, wherein the bacteria is *E. coli*, the bacteria expresses 3-hydroxyacyl-AC P thioesterase and wherein 3-hydroxy acids are secreted into the culture medium, further comprising collecting the 3-hydroxy acids from the medium.

Evidence Appendix

I. Evidence submitted with Information Disclosure Statement filed on May 8, 2002.

Madison and Huisman, *Microbiol and Mol Biol Rev.*, 63(1):21-53 (1999).

Rehm, et al., *J. Biol. Chem.*, 273(37):24044-24051 (1998)

II. Evidence submitted with Response filed on July 10, 2008.

Poirier, Progress Lipid Res. 41:131-155 (2002)

Wikipedia "Posttranslation Modification"

Related Proceedings Appendix

None

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A New Metabolic Link between Fatty Acid *de Novo* Synthesis and Polyhydroxyalkanoic Acid Synthesis

THE *PHAG* GENE FROM *PSEUDOMONAS PUTIDA* KT2440 ENCODES A 3-HYDROXYACYL-ACYL CARRIER PROTEIN-COENZYME A TRANSFERASE*

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To investigate the metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid (PHA) synthesis, we isolated mutants of *Pseudomonas putida* KT2440 deficient in this metabolic route. The gene *phaG* was cloned by phenotypic complementation of these mutants; it encoded a protein of 295 amino acids with a molecular mass of 33,876 Da, and the amino acid sequence exhibited 44% amino acid identity to the primary structure of the *rhlA* gene product, which is involved in the rhamnolipid biosynthesis in *Pseudomonas aeruginosa* PG201. S_1 nuclease protection assay identified the transcriptional start site 239 base pairs upstream of the putative translational start codon. Transcriptional induction of *phaG* was observed when gluconate was provided, and PHA synthesis occurred from this carbon source. No complementation of the *rhlA* mutant *P. aeruginosa* UO299-harboring plasmid pBHR81, expressing *phaG* gene under *lac* promoter control, was obtained. Heterologous expression of *phaG* in *Pseudomonas oleovorans*, which is not capable of PHA synthesis from gluconate, enabled PHA synthesis on gluconate as the carbon source. Native recombinant PhaG was purified by native polyacrylamide gel electrophoresis from *P. oleovorans*-harboring plasmid pBHR81. It catalyzes the transfer of the acyl moiety from *in vitro* synthesized 3-hydroxydecanoyl-CoA to acyl carrier protein, indicating that PhaG exhibits a 3-hydroxyacyl-CoA-acyl carrier protein transferase activity.

Fluorescent pseudomonads belonging to the rRNA homology group I are able to synthesize and accumulate large amounts of polyhydroxyalkanoic acids (PHA)[1] consisting of various saturated 3-hydroxy fatty acids with carbon chain length ranging from 6 to 14 carbon atoms as carbon and energy storage compound (1). PHA isolated from these bacteria contained also constituents with double bonds or with functional groups such as branched, halogenated, aromatic, or nitrile side chains (2). The composition of PHA depends on the PHA synthases, the carbon source, and the involved metabolic routes (2–6). In *Pseudomonas putida* at least three different metabolic routes occur for the synthesis of 3-hydroxyacyl coenzyme A thioesters, which are the substrates of the PHA synthase (7). (i) β-Oxidation is the main pathway when fatty acids are used as carbon source. (ii) Fatty acid *de novo* biosynthesis is the main route during growth on carbon sources that are metabolized to acetyl-CoA, like gluconate, acetate, or ethanol. (iii) Chain elongation reactions in which acetyl-CoA moieties are condensed to 3-hydroxyacyl-CoA is involved in the PHA synthesis during growth on hexanoate. Recently, recombinant PHA_{MCL} (MCL = medium chain length) synthesis was also obtained in a β-oxidation mutant of *Escherichia coli* LS1298 (*fadB*) expressing PHA synthase genes from *Pseudomonas aeruginosa* (8, 9), indicating that the β-oxidation pathway in *E. coli* provides precursors for PHA synthesis (8). From extended homologies of the primary structures of PHA_{MCL} synthases to PHA_{SCL} (SCL = short chain length) synthases (1), which occur in bacteria accumulating poly(3-hydroxybutyric acid) such as *e.g. Alcaligenes eutrophus*, it seems also likely that the substrate of PHA_{MCL} synthases is (*R*)-3-hydroxyacyl-CoA in pseudomonads. The main constituent of PHA of *P. putida* KT2442 from unrelated substrates such as gluconate is (*R*)-3-hydroxydecanoate (7, 10, 11). Thus, to serve as substrate for the PHA synthase, (*R*)-3-hydroxyacyl-ACP must be converted to the corresponding CoA derivative. This can be mediated in a one step reaction by an (*R*)-3-hydroxyacyl (ACP to CoA) transferase. Another possibility is the release of (*R*)-3-hydroxydecanoic acid by a thioesterase, and subsequent activation to the CoA derivative. Only few enzymes have been described catalyzing a similar reaction. Examples are the malonyl-CoA-ACP transferase, which catalyzes the transfer of the malonyl moiety from CoA to ACP (12), and (*R*)-3-hydroxydecanoyl-ACP-dependent UDP-GlcNAc acyltransferase, which catalyzes the transfer of hydroxydecanoyl moeity from ACP to UDP-GlcNAc (13, 14). In this study, we describe the isolation and characterization of *P. putida* KT2440 mutants, which are defective in the PHA synthesis via fatty acid *de novo* biosynthesis, and we identified and characterized the gene locus, which phenotypically complements these mutants. The gene product of *phaG* was purified, and the catalyzed reaction was identified.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF052507.

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[1] The abbreviations used are: PHA, polyhydroxyalkanoic acid; ACP, acyl carrier protein; PAGE, polyacrylamide gel electrophoresis; CDW, cellular dry weight; kbp, kilobase pair(s); ORF, open reading frame; HPLC, high performance liquid chromatography.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth of Bacteria—Pseudomonads and *Escherichia coli* strains as well as the plasmids used in this study are listed in Table I. *E. coli* was grown at 37 °C in Luria-Bertani (LB) medium. Pseudomonads were grown at 30 °C either in nutrient broth complex medium (0.8%, w/v) or in a mineral salts medium with 0.05% (w/v) ammonia (15).

Nitrosoguanidine Mutagenesis—Mutagenesis was performed according to Miller (16). Cells were incubated for 15 min in the presence of 200 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine/ml.

Polyester Analysis—3–5 mg of lyophilized cell material was subjected

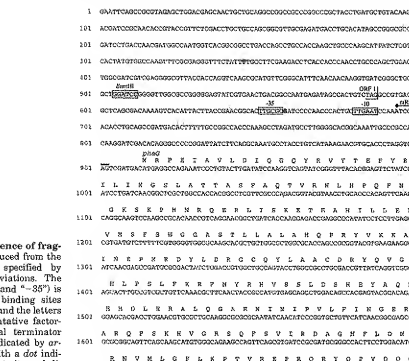

FIG. 1. **Nucleotide sequence of fragment E3.** Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations. The promoter sequence ("−10" and "−35") is *boxed*. Putative ribosome binding sites are indicated by *black bars* and the letters *S/D*. The position of a tentative factor-independent transcriptional terminator downstream of *phaG* is indicated by *arrows*. An *arrow* starting with a *dot* indicates the transcription start site and direction of transcription.

to methanolysis in the presence of 15% (v/v) sulfuric acid. The resulting methyl esters of the constituent 3-hydroxyalkanoic acids were assayed by gas chromatography according to Brandl *et al.* (17) and as described in detail recently (10).

Isolation, Analysis, and Manipulation of DNA—Plasmid DNA was prepared from crude lysates by the alkaline extraction procedure (18). Total genomic DNA was isolated according to Ausubel *et al.* (19). All genetic procedures and manipulations of DNA were conducted as described by Sambrook *et al.* (20). DNA sequencing was carried out by the dideoxy chain termination method (21) with single-stranded or with double-stranded alkali-denatured plasmid DNA but with 7-deazaguanosine 5′-triphosphate instead of dGTP (22) and with α-^{35}S-dATP using a T7 polymerase sequencing kit according to the manufacturer's protocol (Amersham Pharmacia Biotech). Synthetic oligonucleotides were used as primers, and the "primer-hopping strategy" (23) was employed. Analysis was done in 8% (w/v) acrylamide gels in buffer, pH 8.3, containing 100 mM hydrochloride, 83 mM boric acid, 1 mM EDTA, and 42% (w/v) urea in a S2-sequencing apparatus (Life Technologies, Inc.). Nucleic acid sequence data and deduced amino acid sequences were analyzed with the sequence analysis software package (version 6.2, June 1990) according to Devereux *et al.* (24). The nucleotide and amino acid sequence data reported here have been submitted to GenBank™ under accession number AF052507.

Determination of the Transcriptional Start Site—Total RNA was isolated as described by Oelmüller *et al.* (25). The determination of the transcriptional start site was done by a S_1 nuclease protection assay. The hybridization conditions for the S_1 nuclease protection assays were done as described by Berk and Sharp (26) and Sambrook *et al.* (20), and the S_1 nuclease reactions were conducted as described by Aldea *et al.* (27). DNA probes and dideoxynucleotide sequencing reactions for sizing the signals were performed with pBluescript SK⁻BH13 DNA as a template. In the annealing reaction, the oligonucleotide (5′-GGGTAT-TCGCGCTTCACGT-3′) complementary to positions 887 to 871 and the oligonucleotide 5′-CCGCATCCGCCGCGATAG-3′ complementary to positions 986 to 970, respectively, were used for ^{35}S labeling. For all mapping experiments, 25 μg of RNA was mixed with the labeled DNA fragments (10^7 cpm/μg of DNA).

Polymerase Chain Reaction—Polymerase chain reaction amplifications were performed in 100-μl volumes according to Sambrook *et al.* (20) in an Omnigene thermocycler (Hybaid Ltd., Teddington, U. K.) with Vent polymerase (New England Biolabs GmbH, Schwalbach, Germany). The following oligonucleotides were used as primers to amplify the coding region of *phaG* to construct plasmids pBHR-QG (derivative of pQE60 (Qiagen), insertion into *NcoI*/*Bam*HI sites) and pBHR81 (derivative of pBBR1MCS-2 (28), insertion into *Eco*RI/*Bam*HI sites), respectively: 5′-CATGCCATGGGAAGGCCAGAAATCGCTGTA-3′, 5′-

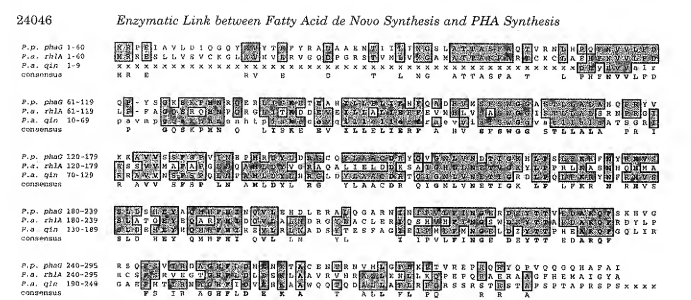

FIG. 2. **Homology of the *phaG* gene product to RhlA (40) and the putative *qin* gene product (GenEMBL data library, accession number L02105) of *P. aeruginosa*.** That part of the amino acid sequence that was deduced from the improved open reading frame analysis of the *qin* nucleotide sequence is given in *lowercase letters*. Matching amino acids are *boxed*. *Dashes* indicate gaps, which were introduced to improve the alignment. *Numbers* indicate the positions of the amino acids in the respective proteins.

CGCGGATCCGATGGCAAATGCATGCTGCCC-3′ (pBHR-QG); 5′-CGGAATTCAAGGAGTCGATGACATG-3′, 5′-CCCGGATCCCGGCGCCCCGTGGCC-3′ (pBHR81). Both plasmids possess artificial ribosome binding sites conserved for *E. coli*, and transcription is regulated by the *lac* promoter.

Preparation of Cell Extracts and Electrophoretic Methods—Approximately 1 g (wet weight) of *E. coli* cells were suspended in 1 ml of buffer A (50 mM Tris hydrochloride, pH 7.4, 0.8% (v/v) Triton X-100, 10 mM $MgCl_2$, 10 mM EDTA, which was supplemented with 200 μg of phenylmethylsulfonyl fluoride per ml) and disrupted by sonification for 1 min at an amplitude of 14 μm in a W 250 sonifier (Branson Schallkraft GmbH, Germany). Soluble cell fractions were obtained as supernatants from 30 min of centrifugation at 50,000 × *g* and 4 °C. SDS- and mercaptoethanol-denatured proteins were separated in 11.5% (w/v) polyacrylamide gels in Tris-glycine buffer (25 mM Tris, 190 mM glycine, 0.1% (w/v) SDS (29) and stained with Coomassie Brilliant Blue (30).

Purification of Recombinant PhaG-His Tag and PhaG—Recombinant PhaG-$(His)_6$ tag (C-terminal fusion) was purified from *E. coli* JM109-harboring plasmid pBHR-QG. Crude extract was subjected to Ni^{2+}-nitrilotriacetic acid-agarose and washed twice with 20 mM imidazole, and the PhaG-$(His)_6$ tag was eluted with 250 mM imidazole. Purified PhaG-$(His)_6$ tag was used to raise anti-PhaG antibodies. Native PhaG was purified from *Pseudomonas oleovorans* ATCC 29347-harboring plasmid pBHR81 by native preparative PAGE (14% (w/v) polyacrylamide) applying the PrepCell 491 (Bio-Rad).

Analysis of (R,S)-3-Hydroxyacyl-CoA or ACP Thioester by High Performance Liquid Chromatography (HPLC)—As a reference substance, (*R,S*)-3-hydroxydecanoyl-CoA was synthesized using 10 milliunits of acyl-CoA synthetase (Sigma) in 100 μl of 50 mM Tris-HCl, pH 7.5, containing 2 mM ATP, 5 mM $MgCl_2$, 2 mM coenzyme A, and 2 mM (*R,S*)-3-hydroxydecanoic acid. The mixture was stopped by the addition of 5 volumes of Dole's reagent (80% (v/v), 20% (v/v) *n*-heptane, 0.02 N H_2SO_4), and remaining free fatty acid was extracted with *n*-heptane. (*R,S*)-3-Hydroxydecanoyl-ACP was synthesized as described by Rock and Cronan were used (31). HPLC analysis was conducted with a RP18 column (nucleosil C18, 7 μm, Knauer) and 25 mM potassium phosphate buffer pH 5.3 as mobile phase. Thioesters were eluted with increasing acetonitrile gradient and detected with a diode array detector (DAD 540, Kontron) at a spectral range of 200 to 500 nm with a 0.8-nm spectral resolution.

Assay of Transfer of 3-Hydroxydecanoate from CoA to ACP—The transferase assay was conducted in 100 μl of 50 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$, 2 mM dithioerythrol, 500 μM acyl carrier protein (Sigma), and 2 mM (*R,S*)-3-hydroxydecanoyl-CoA with a 100-μg protein of crude extract or 50 μg of purified PhaG. After incubation for 4 h at 37 °C, the reaction was stopped by the addition of Dole's reagent, and the reaction mixture was analyzed by HPLC.

RESULTS

Complementation of Mutants Effected in the PHA Synthesis via de Novo Fatty Acid Biosynthesis—Mutants of *P. putida* KT2440, which are only deficient in the metabolic route-linking fatty acid *de novo* synthesis, were generated with nitrosoguanidine according to Miller *et al.* (16). Five mutants ($PhAG_N$) were identified, which accumulated PHA only up to 3% of the cellular dry weight (CDW) from gluconate but up to 85% PHA of CDW when cultivated on octanoate as the sole carbon source. The composition of the polymer was not affected. We constructed a library of *Eco*RI-digested *P. putida* KT2440 genomic DNA with the cosmid vector pVK100 (32) and the Gigapack II Gold Packaging Extract (Stratagene Cloning Systems, La Jolla, CA) in *E. coli* S17-1. Approximately 5,000 transductants were applied to minicomplementation experiments, with mutant $PHAG_N$-21 as recipient. One of the hybrid cosmids (pVK100::K18) harbored three *Eco*RI-fragments (3, 6, and 9 kbp) and enabled $PHAG_N$-21 to accumulate PHA from gluconate. Subcloning revealed that the 3-kbp *Eco*RI fragment (E3, pMPE3) complemented $PHAG_N$-21 and any other $PHAG_N$ mutant exhibiting this phenotype. Complementation was not achieved by the hybrid cosmid pHP1016::PP2000 comprising the entire 7.3-kbp PHA synthase locus of *P. aeruginosa* PAO1 plus approximately 13 kbp of the upstream region or by the hybrid cosmid pHP1016::PP180 comprising the *phaC*2 gene of *P. aeruginosa* PAO1 plus approximately 16 kbp of the adjacent downstream region (10).

Determination of the Gene Locus and Nucleotide Sequence of phaG—Fragment E3 was cloned into pBluescript SK, and the entire nucleotide sequence was determined (Fig. 1). It comprised 3,061 nucleotides with three ORFs (Fig. 1). The only ORF that was completely localized on this fragment was ORF2 with 885 nucleotides starting at position 911 and terminating at position 1795 (Fig. 1). ORF2 will be referred to as *phaG*. A putative S/D sequence was identified eight nucleotides upstream of the start codon. About 230 bp downstream of the translational stop codon a potential factor-independent transcription terminator was located (Fig. 1). ORF1 and ORF3 are localized only incompletely on E3 with ORF1 lacking the 5′-region and with ORF3 lacking the 3′-region. The amino acid sequence deduced from ORF1 revealed significant homologies

TABLE I
Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Source or reference
Strains		
P. putida		
KT2440	mt-2, *hsd*R1 (r^-m^+), ohne TOL plasmid	(45)
$PHAG_{Ny}$-21	*P. putida* KT2440 mutants	This study
P. oleovorans	OCT plasmid	ATCC 29347
P. aeruginosa	Prototroph, Alg^-	ATCC 15692
E. coli		
S17–1	*recA*; harbors the *tra* genes of plasmid RP4 in the chromosome; *proA, thi-1*	(46)
JM109	*recA1 endA1 gyrA96 thi hsdR17* (rk^-,mk^+) *supE44 relA1*, λ-, *lac* [F′*proAB lacI*^{q}ZΔM15]	(21)
Plasmids		
pHP1016::PP180	Te^r, Km^r, $phaC2_{Pu}$ $phaD_{Pu}$, ORF4 orientation of the Cm promoter antilinear to *phaC2*	(47)
pHP1014::PP2000	Te^r, Km^r, $phaC1_{Pu}$, $phaZ_{Pu}$, $ORF1_{Pu}$ ORF2, $phaD_{Pu}$, ORF4′	(47)
pVK100	Te^r, Km^r, broad host range cosmid	(32)
pVK100::K18	pVK100 harboring three genomic *Eco*RI fragments of *P. putida* KT2440 harboring *phaG*	This study
pMP92	Tc^r, broad host range plasmid	(48)
pMPE3	pMP92 containing the 3-kbp E3 fragment harboring *phaG*	This study
pUCP27	Te^r, broad host range plasmid, lacPOZ′	(49)
pBHR75	pUCP27 containing the 1.3-kbp *Bam*HI-*Hin*dIII subfragment of E3 comprising *phaG* including the native promoter	This study
pMPSE22	pMP92 containing the 2.2-kbp *Sal*I-*Eco*RI subfragment of E3 harboring *phaG* without promoter	This study
pBBR1MCS-2	Km^r, broad host range, *lacPOZ′*	(28)
pBHR81	pBBR1MCS-2 containing coding region of *phaG* downstream of *lac* promoter	This study
pBluescript SK$^-$	Ap^r, *lacPOZ′*, T7 and T3 promoter	Stratagene
pBluescript SK$^-$BH13	pBluescript SK$^-$ containing 1.3-kb *Bam*HI-*Hin*dIII subfragment of E3 comprising *phaG* including the native promoter	This study
pQE60	Ap^r, *lacP*, C terminal His tag fusion	Qiagen
pBHR-QG	pQE60 containing coding region of phaG in *NcoI*/*Bam*HI site in-frame to create His tag fusion	This study

to a hypothetical, not further characterized protein of *Haemophilus influenzae* (33). In contrast, the amino acid sequence deduced from ORF3 did not reveal any significant homology to proteins available from EMBL data base. Several other smaller ORFs were detected. However, none of them did obey the rules of Bibb *et al.* (34) for a coding region or was preceded by a reliable ribosomal binding site.

Characterization of the phaG Translational Product—The codon usages in *phaG*, ORF1 and ORF3 agreed well with typical *P. putida* codon preferences. The G + C content of 59.2 mol % for *phaG* was similar to the value of 60.7 to 62.5 mol % determined for total genomic DNA of *P. putida* (35). The *phaG* gene encodes a protein of 295 amino acids with a molecular mass of 33,876 Da. Sequence alignments of the amino acid sequence deduced from *phaG* revealed a 44% overall identity to the *rhlA* gene product of *P. aeruginosa* PG201 (Fig. 2). RhlA also consists of 295 amino acids and has a molecular mass of 32.5 kDa. This gene represents the 5′-terminal gene of a gene cluster consisting of the genes *rhlA*, *rhlB*, and *rhlR*. The first two genes encode proteins involved in rhamnolipid biosynthesis. The *rhlB* gene product exhibited rhamnosyltransferase activity, whereas the function of RhlA is not yet characterized but is necessary for effective rhamnolipid biosynthesis. RhlR represents a transcriptional activator acting upon σ^{54}-dependent promoters (36). The C-terminal regions of RhlA and PhaG revealed high homology to a gene region (*qin*) of *P. aeruginosa* encoding the so-called "quinolone-sensitivity protein" (GenEMBL data library, accession number L02105) amounting to 50.6 and 40.1% to PhaG or to RhlA, respectively, in 249 overlapping residues (Fig. 2). This region comprises 1503 nucleotides. The N terminus of the *qin* gene was not exactly determined, and the homology as depicted in the data base extents only from nucleotide 207 to 566 of this sequence (Fig. 2). However, translation of this sequence in all six reading frames and a subsequent tBLASTn search resulted in the identification of homologies also in the upstream region of the suggested *qin* translational start codons but in different reading frames with the N-terminal region of PhaG and RhlA.

Identification and Regulation of the Promoter—244 bp upstream of *phaG*, a putative σ^{70}-dependent promoter structure **TTGCGCN$_{17}$TTGAAT** (where N is a nucleoside) was identified. The promoter was verified by complementation studies of mutant $PHAG_{Ny}$-21 with subfragments of E3. The 2.2-kbp *Sal*I-*Eco*RI subfragment (SE 22, pMPSE22) (Fig. 1, Table I), which lacked the above-mentioned promoter sequence, did not complement this mutant, whereas the 1.3-kbp *Bam*HI-*Hin*dIII subfragment (BH13, pBHR75) (Fig. 1, Table I) of E3 conferred the ability to again synthesize PHA from simple carbon sources. In addition, the significance of this putative promoter structure was proved by S_1 nuclease protection with total RNA isolated from gluconate-grown and octanoate-grown cells of *P. putida* KT2440 harvested in the stationary growth phase. The transcriptional start site was identified 5 nucleotides downstream of the putative promoter consensus sequence at position 673 (Fig. 1, 3). For octanoate-grown cells only an extremely weak RNA signal was detected, whereas a strong signal occurred with RNA isolated from gluconate-grown cells (Fig. 3). This indicated a strong transcriptional induction of *phaG* under conditions of PHA synthesis via fatty acid *de novo* biosynthesis.

Heterologous Overexpression of phaG in E. coli—A plasmid expressing a C-terminal His(6) tag fusion protein of PhaG was constructed. The resulting plasmid pBHR-QG enabled overexpression of *phaG* under *lac* promoter control in *E. coli* JM109 (Fig. 4). The fusion protein could only be purified under denaturing conditions by immobilized metal ion affinity (Fig. 5) and was used as antigen to raise antibodies.

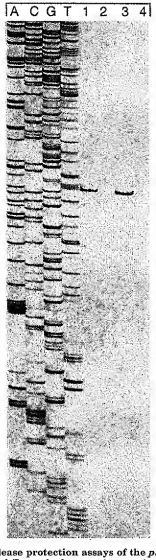

FIG. 3. **S_1 nuclease protection assays of the *phaG* transcripts.** *Lanes A*, *C*, *G*, and *T*, standard sequencing reactions to size the mapping signals. RNA was isolated from gluconate-grown (*lanes 1* and *3*) or octanoate-grown (*lane 4*) cells of *P. putida* KT2440 (*lanes 1* and *3*) and *A. eutrophus* H16 (*lane 2*).

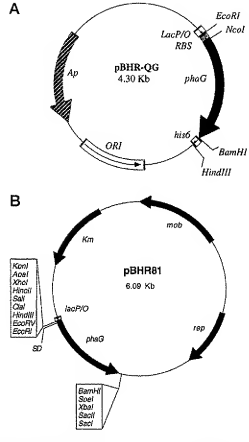

FIG. 4. **Restriction maps of plasmids pBHR-QG (*a*) and pBHR81 (*b*).** *kb*, kilobases.

Functional Homologous and Heterologous Expression of phaG—Functional expression, as revealed by complementation of mutant $PHAG_N21$, was obtained from plasmid pBHR81, a derivative of vector pBBR1MCS-2 (28) containing the coding region of *phaG* in sites *EcoRI*/*Bam*HI (Fig. 4, Table II). Additionally, transfer of pBHR81 into *P. oleovorans* ATCC 29347, which is not capable of PHA synthesis from simple carbon sources, resulted in PHA accumulation from gluconate contributing to about 55% of CDW (Table II). Thus only functional expression of *phaG* in *P. oleovorans* established a metabolic link between fatty acid *de novo* biosynthesis and PHA synthesis. Expression of *phaG* in *P. aeruginosa* PAO1 based on plasmid pBHR81 revealed an ~40% increase in PHA accumulation (Table II). We also investigated functional expression of *phaG* in *E. coli* JM109-harboring plasmids pBHR81 and pBHR71 allowing functional expression of PHA synthase gene *phaC1* (8), but no PHA accumulation was observed when cells were grown on glucose. Furthermore, transfer of pBHR81 into *P. aeruginosa* UO299 (*rhlA*) did not result in complemention of this mutant with respect to rhamnolipid synthesis (data not shown). Thus PhaG does not functionally replace RhlA. To evaluate whether PhaG exhibits PHA synthase activity, we cultivated the *P. putida* $PHAG_N$ mutants harboring pBHR81 under nonlimited nitrogen conditions, which resulted in decreased PHA synthase levels and decreased PHA accumulation (37). No increase in PHA accumulation was observed when cells were grown on gluconate in the presence of PhaG (data not shown).

Enzymatic Assay of PhaG—Native PhaG was purified from crude extracts of *P. oleovorans* (pBHR81) by native PAGE as described under "Experimental Procedures." Recombinant PhaG showed high mobility in native PAGE, which could be utilized for one-step purification (Fig. 5). PhaG was also identified by N-terminal amino acid sequencing.

Purified PhaG and Crude Extracts from *P. oleovorans* (pBHR81) were employed to demonstrate enzymatic activity of PhaG. As substrate we provided *in vitro* synthesized (*R*,*S*)-3-hydroxydecanoyl-CoA and analyzed the reaction products by HPLC (Fig. 6). *P. oleovorans* harboring only vector pBBR1MCS-2 and heat-inactivated purified PhaG served as negative control. The HPLC data clearly demonstrate that, applying either crude extract or purified PhaG, a transfer of the 3-hydroxydecanoyl moeity from CoA to ACP occurs (Fig. 6). The omission of $MgCl_2$ resulted in a loss of enzymatic activity, indicating that $MgCl_2$ is an important cofactor. Furthermore, we applied the straight chain octanoyl-CoA and decanoyl-CoA thioesters as substrate. None of these CoA thioesters yielded the corresponding ACP thioester, and they were therefore not accepted as substrate by PhaG.

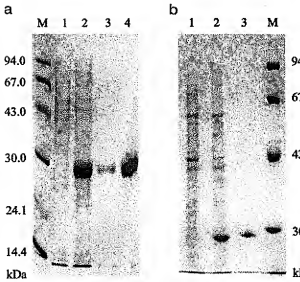

FIG. 5. *a*, heterologous expression of *phaG-His tag* in *E. coli* and purification. Cytoplasmic fractions obtained from cells of recombinant strains of *E. coli* grown in LB medium and fractions from batch purification with Ni^{2+}-nitrilotriacetic acid-agarose were separated in 11.5% (w/v) polyacrylamide gels and stained to visualize protein with Serva blue R. *M*, molecular weight standards. *Lane 1*, crude extract of *E. coli* JM109 (pQE60); *lane 2*, crude extract of *E. coli* JM109 (pBHR-QG); *lane 3*, eluate after washing with 20 mM imidazole; *lane 4*, purified PhaG-His tag after elution with 250 mM imidazole. *b*, heterologous expression of *phaG* in *P. oleovorans* and purification of native PhaG. *P. oleovorans* harboring pBHR81 was cultivated 16 h at 30 °C on mineral salts medium containing 1% (w/v) gluconate. Crude extracts were applied to native PAGE (PrepCell 491, Bio-Rad), and the first fraction with high absorption at 280 nm yielding purified PhaG was analysed. *M*, molecular weight standards. *Lane 1*, crude extract of *P. oleovorans* (pBBR1MCS-2); *lane 2*, crude extract of *P. oleovorans* (pBHR81); *lane 3*, first protein eluate from native PAGE containing pure PhaG.

DISCUSSION

Phenotypical complementation of *P. putida* KT2440 $PHAG_N$ mutants, which are affected in PHA biosynthesis based on fatty acid *de novo* biosynthesis, led to the identification and characterization of *phaG* as a new gene locus relevant for PHA biosynthesis in *P. putida*. The PHA synthesis pathway via β-oxidation was not impaired in the $PHAG_N$ mutants. $PHAG_N$ mutants were not complemented with the PHA synthase locus of *P. aeruginosa* PAO1 and adjacent genomic region. Therefore, $PHAG_N$ mutants are not defective in the PHA synthase locus, and most probably *phaG* is not closely linked to the PHA synthase locus. Furthermore, *phaG* is not in general essential for the synthesis of PHA in *P. putida* KT2440 but is only required for PHA synthesis and accumulation from gluconate or other simple carbon sources, which are catabolized to acetyl-CoA in this organism before PHA synthesis starts.

From results of labeling studies, nuclear magnetic resonance spectroscopy and gas chromatography-mass spectroscopy Eggink *et al.* (4) and Huijberts *et al.* (7, 38) concluded that the precursors of PHA_{MCL} biosynthesis from simple carbon sources are predominantly derived from (*R*)-3-hydroxyacyl-ACP intermediates occurring during the fatty acid *de novo* biosynthetic route. Since the constituents of PHB and PHA represent the *R* configuration, and since PHA_{SCL} and PHA_{MCL} synthases are highly homologous, the intermediates in fatty acid metabolism are presumably converted to (*R*)-3-hydroxyacyl-CoA before polymerization. Nevertheless, some other routes of PHA synthesis are also possible. Other conceivable alternatives are the release of free fatty acids by the activity of a thioesterase with a thiokinase, subsequently activating these fatty acids to the corresponding hydroxyacyl-CoA thioesters or chain elongation with β-ketothiolase, or β-oxidation of synthesized fatty acids. Evidence for the latter pathways in *P. putida* (7) was obtained and explains why *phaG* mutants are not completely defective in PHA_{MCL} biosynthesis from gluconate. Functional expression of either PHA synthase and accumulation of PHA_{MCL} from fatty acids indicate that PHA synthases are not utilizing (*R*)-3-hydroxyacyl-ACP derivatives as substrate (8, 9).

All mutants analyzed and complemented by *phaG* synthesized PHA to some extent (0.5–3% CDW) with a typical monomer composition of polyester derived from simple carbon sources, as far as detectable. However, analysis of mutant complementation studies and the genomic organization of *phaG* revealed no indication for the existence of another protein essential for the PHA synthesis from simple carbon sources in *P. putida* KT 2440. Therefore, most probably only one additional specific enzymatic step is required for PHA synthesis from gluconate that is not required for PHA synthesis from octanoate. This hypothesis was supported by the observation that only PhaG conferred the ability to synthesize PHA from gluconate to *P. oleovorans*, which lacks this capability (Table II). Furthermore, the analysis of enzymatic activity of PhaG strongly suggests that one enzyme is sufficient to link fatty acid *de novo* synthesis with PHA synthesis (Fig. 6). Evidence that PhaG is not directly involved in synthesis of PHA_{MCL} was provided by cultivations of the *P. putida* $PHAG_N$ mutants (pBHR81) under nitrogen limited and nonlimited conditions. Under nonlimited conditions the level of PHA synthases and PHA_{MCL} accumulation is significantly decreased (37), and even in the presence of PhaG, no increase in PHA_{MCL} synthesis was observed.

Although no complementation of rhamnolipid synthesis in *P. aeruginosa rhlA* mutant UO299 was obtained with *phaG* expressed from plasmid pBHR81, the high degree of homology of *phaG* to *rhlA* and the *qin* region of *P. aeruginosa*, respectively, indicates a related function of these proteins. The exact function of the "quinolone sensitivity protein" has not yet been described. Quinolones such as nalidixic acid are synthetic antibiotics exhibiting strong antimicrobial effects on Gram-negative bacteria including *P. aeruginosa*. The *rhlA* gene product is involved in the rhamnolipid biosynthesis of *P. aeruginosa* PG201, which are synthesized as biosurfactants during the late exponential and stationary growth phases. Rhamnolipid biosynthesis proceeds by sequential glycosyl transfer reactions, each catalyzed by specific rhamnosyltransferases with TDP-rhamnose acting as a rhamnosyl donor, and 3-hydroxydecanoyl-3-hydroxydecanoate or L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate acting as acceptors as proposed by Burger *et al.* (39, 40). 3-Hydroxydecanoate can be formed via β-oxidation or via fatty acid *de novo* biosynthesis (41). A dimer consisting of two 3-hydroxydecanoic acid molecules is formed by a hitherto unknown mechanism. RhlA significantly enhanced the level of rhamnolipids in rhamnolipid-negative mutants of *P. aeruginosa* PG201 when it was coexpressed with the rhamnosyltransferase (RhlB) as compared with the expression of the isolated *rhlB* gene.

3-Hydroxyacyl-ACP intermediates provided by fatty acid biosynthesis are presumably the common intermediates of PHA and rhamnolipid biosynthesis from gluconate. If the ACP derivatives themselves do not serve as substrates for PHA synthases or enzymes involved in rhamnolipid synthesis for the condensation of two 3-hydroxydecanoyl moieties, they must be either directly transesterified to the corresponding CoA derivatives or transferred to CoA thioesters by the combined action of a thioesterase and a thiokinase. Various transacylases and acyltransferases have been described and well characterized

TABLE II

Complementation of P. putida mutant PHAG$_N$-21 and functional heterologous expression of phaG in various pseudomonads

PHA content and comonomer composition of various pseudomonads harboring either vector pBBR1MCS-2 or pBHR81. Cells were grown for 48 h at 37 °C (*P. aeruginosa*) or at 30 °C (all others). Cultivations were performed in a mineral salts medium containing 1% (w/v) gluconate. PHA content and comonomer composition were analyzed. 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.

Strain	Plasmid	PHA content	Composition of PHA			
			3HHx	3HO	3HD	3HDD
		% (w/w) CDW	mol %			
P. putida KT2440	pBBR1MCS-2	54	3.1	24.2	66.4	6.3
	pBHR81	60	3.2	14.2	75.1	7.5
P. putida PHAG$_N$-21	pBBR1MCS-2	3	[a]ND[a]	25.3	65	10
	pBHR81	50	3.1	14.2	76.6	6.1
P. aeruginosa PAO1	pBBR1MCS-2	37	2.5	20.5	68	9
	pBHR81	51	2.6	25	60	12.4
P. oleovorans[b]	pBBR1MCS-2	3	ND	ND	75	25
	pBHR81	46	1	7.5	78	13.5

[a] ND, not detectable.
[b] Strain ATCC29347.

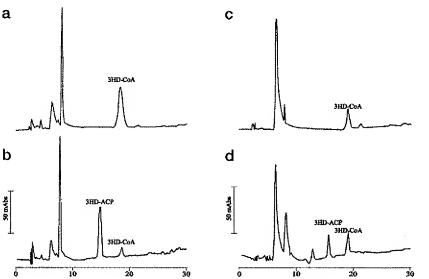

FIG. 6. **HPLC analysis of reaction products from enzymatic assay with PhaG.** *a*, crude extracts from various bacteria harboring either (*a*) vector pBBR1MCS-2 (negative control) or (*b*) plasmid pBHR81 were employed for the enzymatic PhaG assay. *d*, purified PhaG was directly used for the assay (*c*) with heat-inactivated PhaG as negative control. 3-Hydroxydecanoyl-CoA (*3HD-CoA*) was provided as substrate, and the transfer of the acyl moeity to ACP was demonstrated (3-hydroxydecanoyl-ACP (*3HD-ACP*)). Peaks were identified based on their R_f values, by co-chromatography, and by their spectra. The identity of relevant peaks was indicated.

catalyzing the direct transfer of an acyl moiety, *e.g.* (i) the malonyl-CoA-ACP transferase, which catalyzes the transfer of the malonyl moeity from CoA to ACP (12) and (ii) the hydroxydecanoyl-ACP-dependent UDP-GlcNAc acyltransferase, which catalyzes the transfer of hydroxydecanoyl moeity from ACP to UDP-GlcNAc (13, 14). The bacterial acyltransferase LpxA is one representative of a large family that possesses conserved repeating hexapeptides (42). Sequence analysis of membrane-bound glycerolipid acyltransferases revealed that these proteins share a highly conserved domain containing invariant histidine and aspartic acid residues separated by four less conserved residues in an HX_4D configuration (43). Site-directed mutagenesis of the invariant histidine resulted in lack of activity, indicating an essential role of this residue (43). Although no significant homology of PhaG to transacylases and acyltransferases was found, this highly conserved HX_4D mini-motif is also present in PhaG at positions 176–181 of the amino acid sequence (Fig. 1), suggesting a similar function of PhaG. The studies on heterologous expression of *phaG* and the enzymatic characterization of PhaG strongly suggests that PhaG catalyzes the conversion of (*R*)-3-hydroxyacyl-ACP to (*R*)-3-hydroxyacyl-CoA derivatives (Table II, Fig. 6), which serve as ultimate precursors for the PHA polymerization from unrelated substrates in pseudomonads proposed recently (4, 44).

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Review

Polyhydroxyalknoate synthesis in plants as a tool for biotechnology and basic studies of lipid metabolism

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Abstract

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyacids naturally synthesized in bacteria as a carbon reserve. PHAs have properties of biodegradable thermoplastics and elastomers and their synthesis in crop plants is seen as an attractive system for the sustained production of large amounts of polymers at low cost. A variety of PHAs having different physical properties have now been synthesized in a number of transgenic plants, including *Arabidopsis thaliana*, rape and corn. This has been accomplished through the creation of novel metabolic pathways either in the cytoplasm, plastid or peroxisome of plant cells. Beyond its impact in biotechnology, PHA production in plants can also be used to study some fundamental aspects of plant metabolism. Synthesis of PHA can be used both as an indicator and a modulator of the carbon flux to pathways competing for common substrates, such as acetyl-coenzyme A in fatty acid biosynthesis or 3-hydroxyacyl-coenzyme A in fatty acid degradation. Synthesis of PHAs in plant peroxisome has been used to demonstrate changes in the flux of fatty acids to the β-oxidation cycle in transgenic plants and mutants affected in lipid biosynthesis, as well as to study the pathway of degradation of unusual fatty acids.

Keywords: Polyhydroxyalkanoates (PHA); (PHB) Polyhydroxybutyrate; Polyester; Transgenic plants; Metabolic engineering; *Arabidopsis*; Plastid; Rapeseed; Peroxisome; Fatty acid; β-oxidation

Contents

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Nomenclature

ACP	acyl carrier protein
btkB	3-ketothiolase
CaMV	cauliflower mosaic virus
CoA	coenzyme A
DAGAT	diacylglycerol acyltransferase
dwt	dry weight
fwt	fresh weight
g	gram
GC-MS	gas chromatography-mass spectrometry
HB	3-hydroxybutyrate
HV	3-hydroxyvalerate
ilvA	threonine deaminase from *Escherichia coli*
MCL-PHA	medium-chain-length polyhydroxyalkanoate
MFP	multifunctional protein
NMR	nuclear magnetic resonance
PDC	puruvate dehydrogenase complex
PHA	polyhydroxyalkanoate
phaA	3-ketothiolase
phaB	acetoacetyl-CoA reductase
phaC	PHA synthase
PHB	poly(3-hydroxybutyrate)
P(HB-HV)	poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
SCL-PHA	short-chain-length polyhydroxyalkanoate

1. Introduction

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyacids synthesized by a wide variety of bacteria as a carbon reserve and electron sink [1–5]. Although they were first discovered in *Bacillus megaterium* by M. Lemoigne in 1925 [6], it was only several decades later that the value of PHAs as polymers having thermoplastic and elastomeric properties was recognized in a patent application. Since then, the potential application of PHAs as biodegradable, renewable, and environmentally-friendly plastics has been the main driving force behind research on its synthesis in bacteria and its application in polymer chemistry. Over 100 different monomers have been found to be included in bacterial PHAs and the metabolic pathways involved in the synthesis of a variety of these polymers have been elucidated to some degree [7,8]. The chemical diversity of PHA translates into a wide spectrum of physical properties, ranging from stiff and brittle plastics, to softer plastics, elastomers, rubbers and glues [9]. Despite its basic attractiveness as a substitute for petroleum-derived polymers, the major hurdle facing commercial production and application of PHA in consumer products is the high cost of bacterial fermentation, making bacterial PHA 5–10 times more expensive then the petroleum-derived polymers, such as polypropylene and polyethylene, which cost approximately $0.25 to 0.5/kg [10]. It is in this perspective that synthesis of PHA in plants was seen as an attractive proposition [10–12]. Since crops plants can produce annually millions of tons of starch and oils at costs ranging from $0.25-1.0/kg, synthesis of PHA in crops is seen as potentially the only way of producing PHA on a large scale and at low cost.

Synthesis of PHA in plants was first demonstrated in 1992 by the accumulation of poly(3-hydroxybutyrate) (PHB) in the cytoplasm of cells of *Arabidopsis thaliana* [13]. Since then, a range of different PHAs have been synthesized in various species through the creation of novel metabolic pathways either in the cytoplasm, plastid or peroxisome [12]. Although the initial driving force behind synthesis of PHA in plants has been for the biotechnological production of biodegradable polymers, PHA synthesis in plants has more recently emerged as a useful and novel tool to study fundamental aspects of plant metabolism.

This review will focus on the synthesis of PHA in plants. However, since PHA is naturally synthesized in bacteria and most of our knowledge on PHA synthesis and degradation has been obtained from studies in bacteria, a brief review on the main pathways involved in PHA synthesis in bacteria will first be given.

2. Metabolic pathways involved in the synthesis of PHAs in bacteria

PHAs have been shown to occur in over 90 genera of bacteria, encompassing gram-positive and gram-negative species, as well as some cyanobacteria [3,5]. While the majority of PHAs are composed of R-(−)-3-hydroxyalkanoic acid monomers ranging from 3 to 16 carbons in length (C3–C16), some PHAs can also incorporated 4- or 5-hydroxy acids [14] . Well over 100 different hydroxyacids have been found to be incorporated in PHAs, with the major diversity being found in the length and the presence of functional groups in the side chain of the polymer [14]. Although some of these monomers have been found in PHA produced by bacteria in their natural environment, a larger fraction of monomers have been incorporated into PHA following growth of bacteria under laboratory conditions in media containing exotic sources of carbon.

Bacteria synthesizing PHAs have been broadly subdivided in two groups. One group, including the bacterium *Ralstonia eutropha*, produces short-chain-length PHA (SCL-PHA) containing monomers ranging from 3 to 5 carbons in length, while a distinct group, including a number of Pseudomonads, synthesizes medium-chain-length PHA (MCL-PHA) containing monomers ranging from 6 to 16 carbons in length [5]. This division between SCL- and MCL-PHA is mainly determined by the substrate specificity of the PHA synthase responsible for the polymerization of the substrate R-3-hydroxyacyl-CoA to form PHA. It has become clear, however, that this division between SCL- and MCL-PHA is not strict, since several bacteria have been found that can synthesize a "hybrid" PHA that can include monomers from 4 to 8 carbons [15–18].

A number of enzymes and metabolic pathways have been shown to be implicated in the synthesis of a wide spectrum of PHAs in bacteria. In this review we wish to focus only on the pathways which have been transferred into plants. The readers are referred to several excellent recent reviews to learn more on various aspects of bacterial PHA, including biochemical synthesis [2,7,8], degradation in the environment [19,20], fermentation technology [21], and application of PHAs [22].

2.1. Synthesis of polyhydroxybutyrate

PHB is the most widespread and thoroughly characterized PHA found in bacteria. A large part of our knowledge on PHB biosynthesis has been obtained from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) [23]. In this bacterium, PHB is synthesized from acetyl-coenzyme A (CoA) by the sequential action of three enzymes (Fig. 1). The first enzyme of the pathway, 3-ketothiolase, catalyzes the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. Acetoacetyl-CoA reductase subsequently reduces acetoacetyl-CoA to R-(−)-3-hydroxybutyryl-CoA,

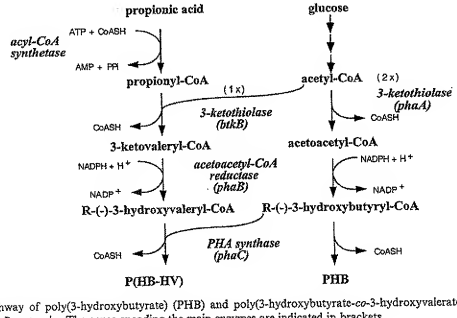

Fig. 1. Pathway of poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-HV)] synthesis in *R. eutropha*. The genes encoding the main enzymes are indicated in brackets.

which is then polymerized by the action of a PHA synthase to form PHB. The PHA synthase *of R. eutropha* has been shown to accept the R-isomer of 3-hydroxybutyryl-CoA but not the S-isomer [24]. PHA is typically produced as a polymer of 10^3 to 10^4 monomers which accumulates as inclusions of 0.2–0.5 μm in diameter. In *R. eutropha*, PHB inclusions can typically accumulate to 80–85% of the dry weight (dwt) when bacteria are grown in media containing excess carbon, such as glucose, but limited in one essential nutrient, such as nitrogen or phosphate [23]. Under these conditions, PHB synthesis acts as a carbon reserve and an electron sink. When growth limiting conditions are alleviated (by addition of phosphate or nitrogen), PHB is depolymerized by the action of an intracellular PHB depolymerase to give acetoacetate, which is then catabolized further to acetyl-CoA [23].

PHB is a highly crystalline polymer and a stiff and relatively brittle thermoplastic [9]. Its melting point (Tm = 175 °C) is only slightly lower then the temperature at which it starts degrading to crotonic acid (approximately 185 °C), making processing difficult. These properties seriously limit its use in a wide range of commodity products. PHB has good ultra-violet light resistance but relatively poor resistance to acids and bases. The polymer is water and air impermeable and relatively resistant to hydrolytic degradation, making it superior to starch-derived plastics which are moisture sensitive.

2.2. Synthesis of poly(hydroxybutyrate-co-hydroxyvalerate)

Because PHB homopolymer has relatively poor physical properties, extensive efforts have been invested on the synthesis of SCL-PHA co-polymers that have better properties. Incorporation of 3-or 5-carbon monomers into a polymer composed mainly of 3-hydroxybutyrate leads to a decrease in the crstallinity and melting point compared to PHB homopolymer [9]. The co-polymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate; PHB-HV) is, thus, less stiff and tougher then PHB, as well as easier to process, making it a good target for commercial application [9]. A number of PHAs with different C3 to C5 monomers have been produced in *R. eutropha*, the nature and proportion of these monomers being influenced by the type and relative quantity of the carbon sources supplied to the growth media [23]. Addition of propionic acid or valeric acid to the growth media containing glucose leads to the production of a random copolymer composed of 3-hydroxybutyrate and 3-hydroxyvalerate P(HB-HV) [23]. The biochemical pathway of P(HB-HV) synthesis from propionic acid is shown in Fig. 1. In *R. eutropha*, condensation of propionyl-CoA with acetyl-CoA is mediated by a distinct 3-ketothiolase, named btkB, which has a higher specificity for propionyl-CoA then the 3-ketothiolase encoded by the *pha*A gene [25]. Reduction of 3-ketovaleryl-CoA to R-3-hydroxyvaleryl-CoA and subsequent polymerization to form P(HB-HV) are catalyzed by the same enzymes involved in PHB synthesis, namely the acetoacetyl-CoA reductase and PHA synthase. While most bacteria synthesize P(HB-HV) only when supplied with an external source of propionic acid or valeric acid, some bacteria can synthesize the same co-polymer when grown only on a simple sugar. One example is *Rhodococcus ruber*, which when grown on glucose produces a copolymer with 75 mol% 3HV and 25 mol% 3HB [26]. The pathway leading to the synthesis of P(HB-HV) in this host has not been completely elucidated. Conversion of succinate to propionyl-CoA through the action of methylmalonyl-CoA mutase and either methylmalonyl-CoA decarboxylase or methylmalonyl-CoA:oxaloacetate transcarboxylase has been proposed to lead to the synthesis of HV units from glucose [27].

2.3. Synthesis of medium-chain polyhydroxyalkanoate

MCL-PHAs are typically described as elastomers, although their actual physical properties are very diverse and are dependent on the monomer composition [9]. Monomers present in MCL-PHA may have a number of functional groups, such as unsaturated bonds and halogenated groups [14]. The presence of reactive groups in the side chain offers opportunities to modify the structure and physical properties of PHAs after extraction. For example, electron-beam irradiation of a MCL-PHA containing unsaturated monomers resulted in the conversion of an initially soft polymer into a cross-linked polymer with properties of a rubber [28].

There are two main routes for the synthesis of MCL-PHA in bacteria [7,23]. The first is the synthesis of PHA using intermediates of fatty acid β-oxidation (Fig. 2A). This pathway is found in several bacteria, such as *Pseudomonas oleovorans* and *Pseudomonas fragii*, which can synthesize MCL-PHA from alkanoic acids or fatty acids. In these bacteria, the monomer composition of the PHA produced is directly influenced by the carbon source added to the growth media. Typically, the PHA is composed of monomers that are 2n ($n \geq 0$) carbons shorter than the substrates added to the media. For example, growth of *P. oleovorans* on octanoate (C8) generates a PHA co-polymer containing C8 and C6 monomers, whereas growth on dodecanoate (C12) generates a PHA containing C12, C10, C8 and C6 monomers [29]. Alkanoic acids present in the media are transported into the cell where they are first converted to CoA esters before being directed to the β-oxidation pathway where a number of 3-hydroxyacyl-CoA intermediates can be generated. Since the PHA synthase accepts only the R isomer of 3-hydroxyacyl-CoA and the bacterial β-oxidation of saturated fatty acids generates only the S isomer of 3-hydroxyacyl-CoA [30,31],

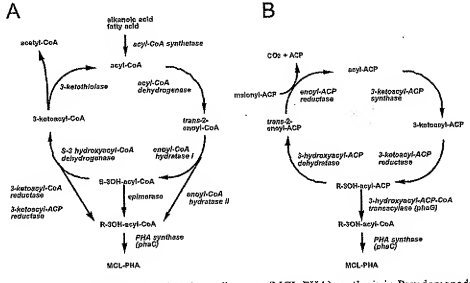

Fig. 2. Pathway of medium-chain-length polyhydroxyalkanoate (MCL-PHA) synthesis in Pseudomonads. (A) Synthesis of MCL-PHA using intermediates of fatty acid β-oxidation intermediates. (B) Synthesis of MCL-PHA using intermediates of fatty acid biosynthesis.

bacteria must have enzymes capable of generating R-3-hydroxyacyl-CoA. One enzyme is a 3-hydroxyacyl-CoA epimerase, mediating the reversible conversion of the S and R isomers of 3-hydroxyacyl-CoA. This enzyme activity is found as a part of the multifunctional protein (MFP), the second enzyme participating in the core β-oxidation cycle. The MFP possesses, in addition to the 3-hydroxyacyl-CoA epimerase activity, an enoyl-CoA hydratase I, S-3-hydroxyacyl-CoA dehydrogenase and a Δ^3-Δ^2-enoyl-CoA isomerase activity [32]. Furthermore, monofunctional enoyl-CoA hydratase II enzymes, converting directly enoyl-CoA to R-3-hydroxyacyl-CoA, have been identified in several bacteria, including *Aeromonas cavea*, *Rhodospirillum rubrum* and *Pseudomonas aeruginosa* [33–36]. Finally, it is speculated that a 3-ketoacyl-CoA reductase that could specifically generate R-3-hydroxyacyl-CoA may exist in bacteria, although such an enzyme has not yet been unambiguously identified. It has, however, been shown that the enzyme 3-ketoacyl-acyl carrier protein (ACP) reductase, participating normally in the fatty acid biosynthetic pathway, may also act on the 3-ketoacyl-CoA to generate R-3-hydroxyacyl-CoA [37], and thus contribute to MCL-PHA synthesis.

The second route for MCL-PHA in bacteria is through the use of intermediates of fatty acid biosynthesis (Fig. 2B). This pathway is also found in numerous Pseudomonads. In contrast to *P. oleovorans* and *P. fragii*, which can only synthesize MCL-PHA from related alkanoic acids present in the growth media, *P. aeruginosa* and *Pseudomonas putida* can synthesize a similar type of MCL-PHA when grown on unrelated substrates, such as glucose [38,39]. Detailed analysis of the composition of PHA produced by *P. putida* grown on glucose revealed the presence of the monomers 3-hydroxy-5-cis-dodecenoic acid and 3-hydroxy-7-cis-tetradecenoic acid [40]. Since these monomers are structurally identical to the acyl-moieties of the R-3-hydroxyacyl-ACP intermediates of the de novo fatty acid biosynthesis, it was hypothesized that these intermediates could be used to form PHAs. This conclusion was further supported by studies using ^{13}C-labeled acetate [41,42]. A key enzyme linking fatty acid biosynthesis and PHA synthesis has first been identified and the corresponding gene cloned from the bacteria *P. putida*. This protein, named phaG, was shown to have a 3-hydroxyacyl-CoA-ACP transferase activity [43]. Expression of the corresponding gene in *P. oleovorans* and *P. fragii* confers to these bacteria the novel capacity to synthesize PHA from glucose [43-45]. Interestingly, a homologue of *pha*G has also been identified in *P. oleovorans*, but was found to be transcriptionally inactive, explaining the inability of this organism to synthesize PHA from fatty acid biosynthetic intermediates [46].

3. Synthesis of polyhydroxyalkanoate in plants

3.1. Synthesis of polyhyhydroxybutyrate in the cytoplasm

Despite its relatively poor physical properties as a thermoplastic, PHB was initially targeted for production in plants because the first bacterial PHA biosynthetic genes that were cloned were for PHB synthesis in the bacterium *R. eutropha* [47–49]. As described in Section 2.1, PHB is synthesized in bacteria from acetyl-CoA. Since acetyl-CoA is present in plant cells in the cytosol, plastid, mitochondrion and peroxisome, the synthesis of PHB in plants could, in theory, be achieved in any of these sub-cellular compartments. However, the cytoplasm was targeted as the first site for PHB synthesis because it had the advantage that the bacterial enzymes could be directly

expressed in this compartment without any modification of the proteins. Furthermore, an endogenous plant 3-ketothiolase is present in the cytoplasm as part of mevalonate pathway. Thus, creation of the PHB biosynthetic pathway in the cytoplasm was theoretically more simple, requiring only the expression of two additional enzymes, the reductase and synthase. The *R. eutropha phaB* and *phbC* genes, encoding, respectively, the acetoacetyl-CoA reductase and PHA synthase, were expressed in plants under the control of the cauliflower mosaic virus (CaMV) 35S promoter, allowing a relatively high expression of the enzymes in a broad range of tissues [13]. Transgenic *A. thaliana* expressing both the PHB synthase and acetoacetyl-CoA reductase were obtained by cross-pollination of plants expressing each of the enzymes. The highest amount of PHB measured in the shoots of these hybrid plants was approximately 0.1% dwt [13]. Detailed analysis of the PHB purified from *A. thaliana* confirmed that that polymer was isotactic poly([R]-(−)-3-hydroxybutyrate) and that the thermal properties of plant PHB were similar to bacterial PHB [50].

Accumulation of PHA granules in the plant cells were visualized by both epifluorescence microscopy of tissues stained with Nile Blue A and by transmission electron microscopy (Fig. 3B) [13]. Both of these methods revealed the presence of granules in all organs of the hybrid transgenic plants, including root, leaf, cotyledon, and seed. The size (0.2–1 μm) and general appearance of PHA inclusions seen by transmission electron microscopy were similar to PHA inclusions found in bacteria. Surprisingly, even though the PHB metabolic pathway was expressed in the cytoplasm, PHB agglomerations were found in several sub-cellular compartments, i.e. cytosol, vacuole and nucleus (Fig. 3B) [13,51]. However, no PHB inclusions were found in plastids and mitochondria. From these results, it was hypothesized that the hydrophobic nature of PHB inclusions might allow them to pass through the single membrane of the vacuole but not the double membrane of organelles such as the plastid and mitochondrion. The nuclear localization of PHB granules may be explained by some affinity of the inclusions to nuclear constituents, leading to their entrapment in the nucleus during cell division. Heterogeneity in the size of the PHB inclusions present in the various organelles was noted, with inclusions found in the nucleus being smaller than inclusions found in the cytoplasm or vacuole [13,51]. It has previously been shown in bacteria that proteins found on the surface of inclusions, named phasins, affect the size of the bacterial granules, presumably by preventing agglomeration [52,53]. It is thus likely that, depending on the sub-cellular compartment, different plant amphiphatic proteins could be

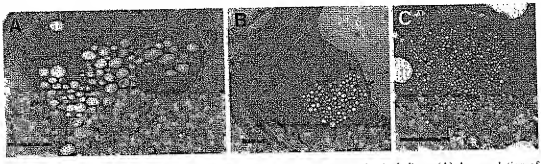

Fig. 3. Accumulation of poly(3-hydroxybutyrate) (PHB) inclusions in transgenic *A. thaliana*. (A) Accumulation of PHB inclusions in the chloroplast of a leaf mesophyll cell expressing the PHB pathway in the plastid. (B) Accumulation of PHB inclusions in the nucleus of a cell expressing the PHB pathway in the cytoplasm. (C) Accumulation of MCL-PHA inclusions in the peroxisome of a cotyledon cell expressing the MCL-PHA synthase in the peroxisome. Bars represent 1 μm.

absorbed on the surface of inclusions, and that these proteins may affect the sizes of the granules through the promotion or prevention of inclusion fusion, in a manner analogous to the bacterial phasins [52,53].

Plants expressing high level of acetoacetyl-CoA reductase have shown a strong reduction in growth, with the most affected plants being approximately five times smaller by fresh weight compared to wild type plants [51]. There was an overall good correlation between the extend of the growth reduction and the level of reductase enzyme activity. It must be noted that plants expressing only the acetoacetyl-CoA reductase do not synthesize PHB. While no abnormal phenotype was observed in plants expressing only the PHB synthase (and not producing PHB), combination of the acetoacetyl-CoA reductase with the PHB synthase led to a further reduction in growth compared to plants expressing only the reductase [13,51]. Although the reasons for the dwarf phenotype has not been unambiguously determined, it was hypothesized that the diversion of cytoplasmic acetyl-CoA and acetoacetyl-CoA away from the endogenous isoprenoid and flavonoid pathways might lead to a depletion of essential metabolites which may affect growth. Since the plant isoprenoid pathway contributes to the synthesis of three classes of plant hormones, namely cytokinins, gibberelins and brassinosteroids, it is likely that even a small imbalance in the synthesis of these hormones may strongly affects plant growth. Cytoplasmic acetyl-CoA and acetoacetyl-CoA are also implicated in the synthesis of sterols, which are essential components of membranes. Interestingly, seeds from plants expressing high levels of acetoacetyl-CoA reductase had a white seed coat, indicating a decrease in the carbon flux towards anthocyanins, at least in the seed coat [51]. The fate of the R-3-hydroxyacyl-CoA produced in the cytoplasm of cells expressing only the acetoacetyl-CoA reductase is unknown. It is possible that this metabolite may itself be toxic to plant cells since it is not expected to be found in plant cell cytoplasm. It is also possible that at least a portion of this CoA ester may be recycled through the peroxisomal β-oxidation pathway.

Synthesis of PHB in plant cell cytoplasm has also been demonstrated in rape and tobacco. Expression of the PHB pathway in the cytoplasm of cells of *Brassica napus* gave results similar to experiments in *A. thaliana*. Cross-pollination of transgenic rape expressing the acetoacetyl-CoA reductase with plants expressing the PHB synthase, both genes expressed under the CaMV 35S promoter, led to hybrids producing PHB in the range 0.02–0.1% of the dry weight (P.A. Fentem, unpublished data). As seen for *A. thaliana*, transgenic rapeseed expressing high amounts of acetoacetyl-CoA reductase were also stunted in growth. Interestingly, overexpression of the bacterial 3-ketothiolase in plants expressing the reductase and PHB synthase did not lead to a significant increase in PHB accumulation, indicating that 3-ketothiolase activity was probably not limiting PHB synthesis in the cytoplasm, but rather that other factors, such as the low flux of acetyl-CoA, may be important.

PHB synthesis has also been demonstrated in the cells of cotton fibers [54,55]. In this approach, PHA is not produced as a source of polyester to be extracted and used in the plastic industries, but rather as an intracellular agent that modifies the heat exchange properties of the fiber. The *pha*A, *pha*B and *pha*C genes from *R. eutropha* were expressed in transgenic cotton under the control of a fiber specific promoter [54,55]. PHB accumulated in the cytoplasm to 0.3% dwt of the mature fiber, a level similar to PHB production in *A. thaliana* cell cytoplasm.

Synthesis of PHB has been demonstrated in *Nicotiana tabacum* through the co-expression of the *pha*B gene from *R. eutropha* and the PHA synthase from *A. caviae* [56]. Although the bacterial

genes were expressed under the strong promoter CaMV35S, expression of both proteins was relatively low and the amount of PHB detected in leaves was only 10 μg/g fwt. Similar results were obtained by the expression of the *R. eutropha* genes in potato (Bohmert et al., personal communication). Poor expression of protein derived from the transcription of *R. eutropha* genes have been previously observed in tobacco (Poirier and Nawrath, unpublished data). The reason for this phenomenon is unknown but may be related to differences in codon usage between Solanaceae species and *R. eutropha* genes, inefficient translation of the mRNA due to an unusual secondary structure, or to an unexplained instability of the proteins.

Detailed analysis of transgenic plants expressing the acetoacetyl-CoA reductase by itself, or in combination with the PHB synthase, may provide valuable insight on a number of aspects of plant metabolism, in particular on flavonoid and isoprenoid biosynthesis in the cytoplasm, two pathways relying on acetyl-CoA. For example, it would be useful to know how the carbon flux to these two pathways is affected in transgenic plants expressing the reductase, as well as how the plant responds to potential disturbances in these pathways by altering the expression of genes. The powerful tools of cDNA microarrays and Affimetrix™ gene chip technology, as well as metabolic profiling, would enable such detailed studies to be undertaken.

3.2. Synthesis of polyhydroxyalkanoate in the plastid

3.2.1. Synthesis of polyhydroxybutyrate in the chloroplasts of Arabidopsis thaliana and corn

Plants expressing the PHB pathway in the cytoplasm accumulated polymer to 0.1% dwt [13,51], which is approximately 200–400 times lower than lipid accumulation in seeds of oil crops, such as rape or soybean, and 800–900 times lower than PHB accumulation in *R. eutropha*. In view of the hypothesis that the limited supply of cytoplasmic acetyl-CoA was thought to be the main factor limiting PHB accumulation and causing reduction in plant growth in the first-generation transgenic plants, expression of the PHB pathway in a compartment with a higher flux through acetyl-CoA was thought to be a potential solution. Fatty acid biosynthesis in plants occurs primarily in the plastid. Thus, this organelle is a site with a large flux of carbon through acetyl-CoA, particularly in tissues having a high proportion of lipids, such as seeds of plants accumulating triacylglycerols as the main carbon reserve. The large flux of acetyl-CoA in the plastids was therefore hypothesized to allow a significantly higher production of PHB while minimizing potential deleterious effects on plant growth. Furthermore, since PHB inclusions synthesized in the cytoplasm were not found within the plastids, it was expected that PHB synthesized in the plastid would not leave the organelle, thus preventing potential disruption of other sub-cellular structures by the movement of inclusions within the cell. Finally, it was noted that since plastids can accumulate a high amount of starch granules without causing disruption of the organelle, the accumulation of PHA inclusions in the plastid may be benign.

In order to express the PHB biosynthetic pathway in plastids of *A. thaliana*, the *R. eutropha* phaA protein, encoding the 3-ketothiolase, as well as phaB and phaC proteins, were modified for plastid targeting by addition of the transit peptide of the small subunit of the ribulose bisphosphate carboxylase from pea [57]. The modified bacterial genes were individually expressed in *A. thaliana* under the control of the constitutive CaMV 35S promoter. Transgenic plants expressing the plastid-targeted reductase were crossed with plants expressing the plastid-targeted PHA synthase. The resulting hybrids did not produce detectable PHB, providing further evidence that

plastids do not have an endogenous 3-ketothiolase activity that could support PHB synthesis [57]. The reductase-synthase double hybrids were subsequently crossed with plants expressing the plastid-targeted 3-ketothiolase to obtain triple hybrids that were producing PHB [57]. Transmission electron microscopy revealed that PHB inclusions accumulated exclusively in the plastids, with some organelles having a substantial portion of their volume filled with inclusions. The size (0.1–0.2 um) and the general appearance of these inclusions were similar to bacterial PHA inclusions (Fig. 3A) [57]. Interestingly, the quantity of PHB in these plants was found to gradually increase over time, with fully expanded pre-senescing leaves typically accumulating 10 times more PHB than young expanding leaves of the same plant. The maximal amount of PHB detected in pre-senescing leaves was 10 mg/g fwt, representing approximately 14% dwt. In contrast to PHB synthesis in the cytoplasm, expression of the PHB biosynthetic enzymes in the plastid was not accompanied by a large reduction in growth of these plants. However, leaf chlorosis was observed in plants accumulating more than 3 mg/g fwt. These results indicated that although the plastid can accommodate a higher production of PHB with minimal impact on plant growth compared to the cytoplasm, there was nevertheless a limit above which alteration in some of the chloroplast functions could be detected [57].

Although successful, the strategy chosen by Nawrath et al. [57] of generating transgenic lines expressing only one transgene and combining the genes though cross-fertilization was labor intensive and made it very difficult to control the copy number of all genes in subsequent generations and to obtain homozygous plants. Furthermore, instability in the expression of the transgenes could be observed in the progeny of the double or triple hybrids [57,58]. It is speculated that the redundancy in the promoter and sequence of the transit peptide used for all three constructs led to the appearance of gene silencing in the hybrids. Thus, in an effort to at least partially avoid these difficulties, a strategy was devised to combine all three genes for PHB biosynthesis on a single vector [58,59]. One such vector was made using the same *pha*A, *pha*B and *pha*C genes that have been modified by Nawrath et al. [57] for targeting the protein to the plastids. Following transformation of this complex vector in *A. thaliana*, a GC-MS method was used to rapidly screen a large number of transgenic lines and to isolate the individuals accumulating high amounts of PHB [59]. By this approach, a number of lines were identified which accumulated between 3 and 40% dwt PHB. While in a line accumulating 3% dwt most of the plastids contained some PHB inclusions, all plastids of mesophyll cells were packed with inclusion in the line containing 40% dwt PHB. Interestingly, these transgenic plants showed a negative correlation between PHB accumulation and plant growth. While plants containing 3% dwt showed only a relatively small reduction in growth, plants accumulating between 30 and 40% dwt PHB were dwarf and produced no seeds [59]. As previously observed by Nawrath et al. [57], all plants producing above 3% dwt PHB showed some chlorosis.

The results of Bohmert et al. [59] and Mitsky et al. [58] are important since they showed that while it is possible to further increase PHB production, the approach of synthesizing PHB in the chloroplasts of shoots has its limits. Furthermore, these experiments demonstrated that the use of multi-gene vectors lead to the production of higher level of PHB then the use of single-gene vectors followed by crossing. The usefulness of such multi-gene vectors is likely to go beyond PHA synthesis to include the expression of numerous novel metabolic pathways in plants requiring the expression of several enzymes. The reasons behind the improvement caused by the use of multi-gene vectors is unclear at present. It is possible that having all transgenes participating in a new

metabolic pathway being genetically linked at a single locus is more favorable due to a better coordination of gene expression. The close proximity of several copies of the promoter may lead to locally high levels of transcription factors, allowing higher expression of the genes. It is also possible that having the genes tightly linked may reduce the incidence or level of gene silencing [58].

PHB synthesized in plants is not thought to be degraded, since significant hydrolysis of PHA requires the presence of specialized bacterial enzymes, the PHA depolymerases [19,20]. PHA in plants is thus viewed as a final and largely un-recyclable carbon sink. This opens several interesting questions about how plants can cope with a new carbon sink. For example, how does PHB synthesis in the plastids affect carbon flow to other compounds synthesized in the organelle, such as starch and fatty acids? How does the plant adjust, at the metabolic and genetic levels, to accommodate for the synthesis of this new sink? Why are plants producing high amount of PHB affected in their growth? Again here, the tools of genomics, proteomics and metabolic profiling could provide interesting answers to these questions and give general insights on plant biochemistry that would go well beyond PHA synthesis in plants.

In a first small-scale study of metabolite profiling, over 60 metabolites (16 fatty acids, 16 sugars and sugar alcohols, 15 amino acids, 12 organic acids and inorganic phosphate) were measured on transgenic *A. thaliana* lines producing high amount of PHB [59]. Surprisingly, no changes in fatty acids were observed. There was, however, a correlation between an increase in PHB with a decrease in levels of isocitrate and fumarate. This may indicate a reduction in tricarboxylic acid cycle activity, leading perhaps to a reduction in pools of acetyl-CoA which may result in growth retardation. There was also a positive correlation between PHB accumulation and levels of several sugars such as mannitol, glucose, fructose and sucrose. Together, these data indicate that a high amount of accumulation of PHB in chloroplasts has a negative effect on plant metabolism. While some of these effects can be explained, such has the decrease in tricarboxylic acid intermediates due to the demand on acetyl-CoA, others are more difficult to interpret. At the gene expression level, no correlation could be found between level of expression of the three *phb* genes and PHB accumulation, leaving unresolved the question of what limits PHB synthesis in the plastids [59].

Scientists at Monsanto have also demonstrated the production of PHB in the plastids of corn leaves and stalk. In these experiments, the same bacterial genes modified for PHB production in the plastids of *A. thaliana* and *B. napus* were used but the constructs also included the HSP70 intron designed to enhance expression in monocots [60]. Various promoters were tested to drive gene expression, including the CaMV 35S and the promoter for the maize chlorophyll A/B binding protein. Levels of PHB accumulation up to 5.7% dwt were reported [60]. Similar to results obtained in *A. thaliana*, there was a progressive accumulation of PHB with time, with older leaves having more polymer than younger leaves. Furthermore, like *A. thaliana*, there was a correlation between leaf chlorosis and higher amount of PHB [60].

Perhaps one of the most striking observations made from the experiments in corn was the fact that while the leaf mesophyll cells showed few PHB granules, the bundle sheath cells associated with the vascular tissue were packed with granules [60]. This is shown quite dramatically in the electron micrograph shown in Fig. 4. This unequal distribution of PHB was not due to the promoter used, since similar pattern was seen for plants transformed with either the CaMV 35S and the chlorophyll A/B binding protein promoters, the latter promoter being known to be a strong promoter in mesophyll cells. Interestingly, similar observation had been made by the Monsanto group for *A. thaliana* plants transformed with the *phb* genes driven by the CaMV35S promoter,

i.e. significantly more granules found in cells surrounding the vascular tissue and epidermal cells compared to mesophyll cells [60]. These results suggest that the availability of plastidial acetyl-CoA for PHB synthesis may be quite different in various cell types, perhaps due to metabolic channeling.

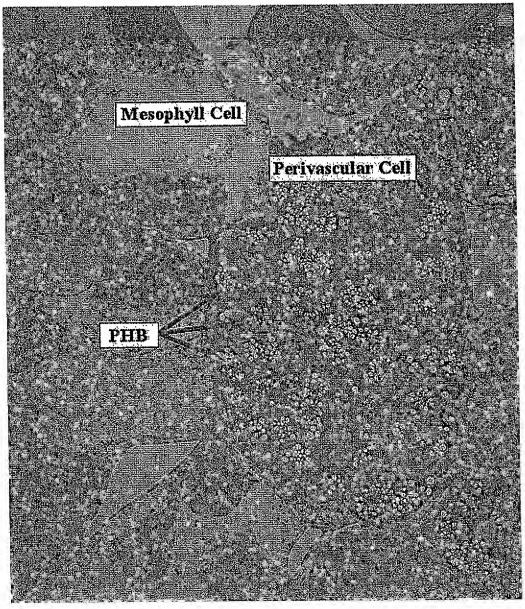

Fig. 4. Accumulation of poly(3-hydroxybutyrate) (PHB) inclusions (arrows) in transgenic corn expressing the biosynthetic pathway in plastids. Note the greater abundance of PHB inclusions in the plastids of the perivascular cell compared to the surrounding mesophyll cells. Photograph from Ken J. Gruys, Monsanto (St-Louis).

3.2.2. Synthesis of polyhydroxybutyrate in the leukoplasts of Brassica napus

In view of the potential limitation of producing PHB in the chloroplast of green tissues, such as leaves, it has been an important issue to evaluate the synthesis of PHB in leukoplast of seeds of an oil crop. This has been accomplished by scientists at Monsanto who reported the successful production of PHB in oilseed leukoplasts of *B. napus* [61,62]. The transformation of *B. napus* was accomplished using a multi-gene vector that contained the entire PHB biosynthetic pathway targeted to the leukoplast. In these experiments, the three modified bacterial genes *pha*A, *pha*B and *pha*C were put under the control of the fatty acid hydroxylase promoter from *Lesquerella fendeleri*, enabling strong expression to the developing seed [63]. PHB levels up to 7.7% fwt of mature seeds were reported [61]. Analysis of seeds by transmission electron microscopy revealed that PHB accumulated exclusively within the leukoplast, and that apparently every visible plastid contained the polymer. Furthermore, the size of the leukoplast was larger in PHB-producing seeds compared to non-transformed seed. This is reminiscent to the enlargement of amyloplast accumulating starch during seed development and suggest that leukoplasts can adjust their size to accommodate novel inclusions.

Seeds accumulating nearly 8% dwt PHB appeared normal and germinated at the same rates as non-transformed seeds [61]. These results demonstrate that at least in the range 3–8% dwt PHB, the seed leukoplast appears a better production system then the leaf chloroplast. It is unknown at this point what is the upper limit of PHB accumulation in seeds and at what level PHB synthesis will start affecting the accumulation of lipids or proteins in the seed, two key factors that have a strong impact on the viability of this approach in the biotechnological production of PHA in oilseed crops (Section 4).

3.2.3. Synthesis of poly(hydroxybutyrate-co-hydroxyvalerate)

PHB is a polymer with relatively poor physical properties, being too stiff and brittle for its use in most consumer products [9]. PHB is, thus, not an ideal polymer for commercial production in transgenic crops. As described previously, the co-polymer P(HB-HV) has lower cristallinity, is more flexible and less brittle than PHB homopolymer [9]. For a number of years, bacterial production of P(HB-HV), also known under the trade name Biopol™, has been central to the marketing and production strategies of PHA by Zeneca and Monsanto. It was therefore natural that after the demonstration of PHB synthesis in several plants, efforts would be focused on the synthesis of PHA co-polymers, such as P(HB-HV).

As described in Section 2.2, synthesis of P(HB-HV) in the bacterium *R. eutropha* relies on the production of propionyl-CoA. It was, thus, necessary to create an endogenous pool of propionyl-CoA in plants which could be used by the PHA pathway. Furthermore, since the plastid was shown to be the best sub-cellular compartment for the synthesis of PHB from acetyl-CoA, it was also chosen as the site for P(HB-HV) synthesis from acetyl-CoA and propionyl-CoA. Although several metabolic pathways exist in prokaryotes and eukaryotes that can generate propionyl-CoA, the simplest strategy adopted by Slater et al. [64] was the conversion of 2-ketobutyrate to propionyl-CoA by the pyruvate dehydrogenase complex (PDC), an enzyme naturally located in the plastid. Although PDC normally decarboxylates pyruvate to give acetyl-CoA, experiments had previously shown that the same enzyme can also decarboxylate 2-ketobutyrate, albeit at low efficiency, to give propionyl-CoA [65]. Since 2-ketobutyrate is also found in the plastid as an intermediate in the synthesis of isoleucine from threonine, both the substrate and the enzyme

complex required for the generation of propionyl-CoA are present in this organelle. However, since PDC would have to compete for the 2-ketobutyrate with the acetolactate synthase, an enzyme involved in isoleucine biosynthesis, the quantity of 2-ketobutyrate present in the plastid was enhanced through the expression of the *E. coli ilv*A gene, which encodes a threonine deaminase (Fig. 5).

The genes encoding the *E. coli ilv*A, the *R. eutropha pha*B and *pha*C, as well as the *bktB* gene from *R. eutropha* encoding a novel 3-thiolase having high affinity for both acetyl-CoA and propionyl-CoA [25], were all modified to add a plastid leader sequence to the enzymes. All genes were expressed under the control of the CaMV35S promoter. Constitutive expression of the ilvA protein along with bktB, phaB and phaC proteins in the plastids of *A. thaliana* lead to the synthesis of P(HB-HV) in the range 0.1–1.6% dwt, and with the fraction of HV units being between 2 and 17 mol% [64].

Expression of the P(HB-HV) pathway in the leukoplast of *B. napus* seeds has also been achieved by putting the bacterial genes under the control of the seed-specific promoter from the *Lesquerella* hydroxylase gene. In these experiments, an isoleucine-insensitive mutant of the *ilva* gene was co-expressed along with the *bkt*B, *pha*A and *pha*C genes, and all four genes were inserted in a single multi-gene vector. P(HB-HV) synthesis in the range 0.7–2.3% dwt was reported, with an HV content of 2.3–6.4 mol% [64]. Interestingly, there was an inverse relationship between the amount of PHA and the proportion of the HV monomer, indicating a bottleneck in providing 3-hydroxyvaleryl-CoA to the PHA synthase. This bottleneck is thought to be caused by the inefficiency of the PDC in converting 2-ketobutyrate to propionyl-CoA. Synthesis of P(HB-HV) in the plastids represent one of the most complex metabolic engineering done in plants, requiring the expression of four transgenes and implicating the diversion of carbon from two sources, namely acetyl-CoA from fatty acid biosynthesis and propionyl-CoA from amino acid biosynthesis.

The impact of P(HB-HV) on plant metabolism was analyzed in more detail for amino acids. In addition of the expected increase in the amount of 2-ketobutyrate, expression of the *E. coli ilv*A

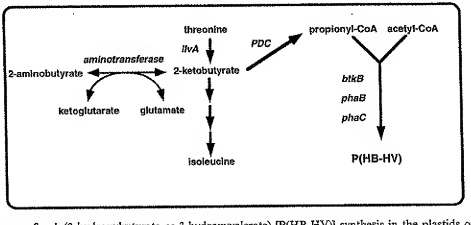

Fig. 5. Pathway of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-HV)] synthesis in the plastids of transgenic plants. A fraction of the 2-ketobutyrate synthesized from the overexpression of the *E. coli ilv*A gene is converted to propionyl-CoA via the plant endogenous pyruvate dehydrogenase complex (PDC). Propionyl-CoA and acetyl-CoA are then used by the enzymes encoded by the *R. eutropha* genes *bkt*B, *pha*B and *pha*C to form P(HB-HV).

threonine deaminase also increased the levels of free isoleucine from 5 to 10 fold [64]. This is due to the fact that threonine deaminase is a committed and regulated step in the biosynthesis of isoleucine. Furthermore, a 17-fold increase in the concentration of 2-aminobutyrate was measured as a result of transamination of 2-ketobutyrate. Expression of the bacterial *ilv*A, thus, resulted in more carbon accumulating in the form of isoleucine or 2-aminobutyrate as compared to the 2-ketobutyrate, the key target for propionyl-CoA biosynthesis. No changes in the levels of either threonine or aspartate were observed, indicating that threonine biosynthesis in plants can compensate for a large increase in flux created by the enhanced levels of ilvA [64].

3.2.4. Synthesis of other polyhydroxyalkanoates in the plastid

As described in Section 2.3, some bacteria can synthesize PHA containing 3-hydroxyacid monomer that are derived from the 3-hydroxyacyl-ACP intermediates of fatty acid biosynthesis. In this pathway, the enzyme phaG plays a key role, catalyzing the conversion of R-3-hydroxyacyl-ACP to R-3-hydroxyacyl-CoA [43], the later being the substrate for the PHA synthase. The identification and cloning of the *P. putida pha*G gene opened the possibility synthesizing PHA co-polymers in the plastids of plants from intermediates of fatty acid biosynthesis. Unfortunately, constitutive expression in the plastid of *A. thaliana* of only the phaG enzyme lead to a marked deleterious effect on plant growth, the plants being dwarf with crinkly leaves and the seed set being strongly reduced (V. Mittendorf, unpublished results). The reason for this phenotype is not known but is thought to be perhaps due to interference of the transacylase with fatty acid biosynthesis. If this is the case, it would be interesting to know why this does not occur in bacteria expressing phaG. Co-expression in the plastid of the *P. aeruginosa* PHA synthase along with phaG did not conclusively lead to PHA accumulation (V. Mittendorf, unpublished results). Thus, despite the obvious advantages of the plastid as a location for the production of PHB and P(HB-HV), the synthesis in this organelle of PHA co-polymer using fatty acid biosynthetic intermediates appears problematic at present.

3.3. Synthesis of polyhydroxyalkanoate in the peroxisome

3.3.1. Synthesis of polyhydroxybutyrate

Acetyl-CoA, the building block of PHB biosynthesis, is found not only in the cytoplasm and plastids, but also in the mitochondria and peroxisomes, being primarily implicated in these organelles in the tricarboxylic acid and β-oxidation cycles, respectively. Although no conclusive demonstration of PHB in plant mitochondria has been reported, synthesis of PHB in the peroxisome was demonstrated in transgenic Black Mexican Sweet corn suspension cell cultures [66]. In these experiments, the *pha*A, *pha*B and *pha*C genes from *R. eutropha* were modified in order to add the amino acids RAVARL at the carboxy terminal end of each protein. The terminal tripeptide ARL is a type I peroxisomal targeting signal and has previously been shown to localize the enzyme glycolate oxidase to the peroxisome of tobacco [67]. Biolistic transformation of maize suspension culture with a mixture of all three genes lead to the isolation of transformants expressing all three enzyme activities and accumulating up to 2% dwt PHB [66]. No detailed effects of peroxisomal PHB biosynthesis on plant metabolism has been reported. As no transgenic plants have been obtained from these transformed cells, it is difficult at this point to evaluate the potential effects of PHB synthesis in peroxisome on growth and metabolism.

3.3.2. Synthesis of polyhydroxyalkanoate from intermediates of fatty acid β-oxidation

MCL-PHA has been shown to be synthesized in *A. thaliana* [68]. The approach used was to divert the 3-hydroxyacyl-CoA intermediates of the β-oxidation of endogenous fatty acids for MCL-PHA production. Since in plants β-oxidation occurs principally in the peroxisomes, PHA biosynthetic proteins needed to be targeted to this organelle. The phaC1 synthase from *P. aeruginosa* was thus modified at the carboxy-end by the addition of the last 34 amino acids from the peroxisomal protein isocitrate lyase of *B. napus*, which harbor a type I peroxisomal targeting sequence. Addition of these sequences to chloramphenicol acetyl transferase had been shown to be sufficient to target the foreign protein to the peroxisome [69]. The modified *phaC1* gene was expressed under the control of the CaMV35S promoter and transformed into *A. thaliana* [68]. Appropriate targeting of the PHA synthase in plant peroxisomes was demonstrated by immunolocalisation. Transmission electron microscopy also showed the presence of electron-lucent inclusions within the peroxisomes (Fig. 3C). These inclusions had an appearance similar to bacterial PHA granules. Furthermore, similar to the enlargement of the leukoplasts of *B. napus* embryos accumulating PHB [61], the peroxisomes of MCL-PHA-producing plants were significantly enlarged compared to peroxisomes of wild type plants. The monomer composition of the MCL-PHA produced in plants reflected well the broad substrate specificity of the PHA synthase of *P. aeruginosa*. Thus, peroxisomal PHA was composed of over 14 different monomers, including saturated and unsaturated monomers ranging from 6 to 16 carbons (Table 1) [68]. The majority of 3-hydroxyacids found into plant MCL-PHA could be clearly linked to the corresponding 3-hydroxyacyl-CoA generated by the β-oxidation of saturated and unsaturated fatty acids. The production of peroxisomal MCL-PHA was relatively low, with a maximal level of 0.4% dwt in 7-day-old germinating seedlings. In leaves, PHA level decreased to approximately 0.02% dwt. This decrease is not thought to reflect PHA degradation but rather the fact that in expanding green tissues the plant weight increases faster then the rate of PHA synthesis. Interestingly, a 2- to 3-fold increase in PHA was observed during leaf senescence. These data support the link between β-oxidation and PHA synthesis, since this pathway, in association with the glyoxylate cycle, are most active during germination and senescence where they are involved in the conversion of fatty acids to carbohydrates. In contrast to PHB synthesis in the cytoplasm and plastid, no negative effects of peroxisomal MCL-PHA accumulation on plant growth or seed germination were observed [68].

Table 1
Composition of polyhydroxyalkanoate (PHA) produced in wild type plant and a mutant affected in fatty acid desaturases

	Monomer composition (mol%)[a]														
	H6	H8	H8:1	H10	H12	H12:1	H12:2	H14	H14:1	H14:2	H14:3	H16	H16:1	H16:2	H16:3
wild type	1.3	19	22	5.3	4.7	3.4	1.4	4.8	3.0	6.3	15	3.5	0.3	3.6	7.4
fad3/fad7-1/fad8	1.4	44	tr	5.5	4.1	11	tr	4.3	2.6	14	0.11	3.7	0.3	8.9	tr

[a] Monomer composition of medium-chain-length polyhydroxyalkanoate (MCL-PHA) isolated from 14-day-old transgenic seedlings. The prefix H denotes 3-hydroxyacid monomers and tr indicates trace. Adapted from [71].

Similar to the PHA synthase from *R. eutropha*, the PHA synthase of *P. aeruginosa* is thought to accept only the R-isomer of 3-hydroxyacyl-CoAs. The wide range of monomers found into plant MCL-PHA suggests that, as with bacteria, plants also have enzymes capable of converting the β-oxidation intermediates S-3-hydroxyacyl-CoA to the R isomer (Fig. 6). Such enzymes could be either the 3-hydroxyacyl-CoA epimerase present on the plant MFP [32] or an enoyl-CoA hydratase II activity which is specific for the generation of R-3-hydroxyacyl-CoA from *trans*-2-enoyl-CoA [70]. A third route for the synthesis of a narrow range of R-3-hydroxyacyl-CoA is the hydration of *cis*-2-enoyl-CoA by the enoyl-CoA hydratase I activity [31]. The substrate *cis*-2-enoyl-CoA is derived from the β-oxidation of unsaturated fatty acids having a *cis* double bond at an even position, such as found in linoleic and linonelic acid.

The MCL-PHA synthesized in *A. thaliana* contained a relatively high proportion of monomers larger then 10 carbons, as well as high proportion of unsaturated monomers (Table 1). From similar PHA produced in bacteria, it is expected that such a polymer would have a low melting point and behave like glue at room temperature. In order to improve the properties of the MCL-PHA produced in plant peroxisome to make it more similar to elastic polymers, it was necessary to decrease the proportion of unsaturated bonds and of longer-chain monomers. Furthermore, commercial exploitation of plants for MCL-PHA synthesis would require amounts of PHA of the order of 10–15% dwt. It was, thus, important to find ways of modulating the quantity and monomer composition of the MCL-PHA synthesized in plant peroxisomes. This was first achieved by influencing the nature and quantity of fatty acids that were targeted to the β-oxidation cycle (Fig. 6) [71].

Growth of transgenic plants in liquid media supplemented with detergents containing various fatty acids was used as a way of increasing the flux of a specific fatty acid to the β-oxidation cycle. Addition of external fatty acids to plants resulted in both an increased accumulation of MCL-PHA and a shift in the monomer composition that reflected the intermediates generated by the β-oxidation of the external fatty acids [71]. For example, addition to the media of the detergent polyoxyethylenesorbitan esterified to lauric acid (Tween-20) resulted in a 8- to 10- fold increase in the amount of PHA synthesized in 14-day-old plants compared to plants growing in the same media without detergent. The monomer composition of the MCL-PHA synthesized media containing Tween-20 showed a large increase in the proportion of saturated even-chain monomers with $\leqslant 12$ carbons, and a corresponding decrease in the proportion of all unsaturated monomers. This shift in monomer composition is accounted by the fact that β-oxidation of lauric acid, a 12 carbon saturated fatty acid, gives saturated 3-hydroxyacyl-CoA intermediates of 12 carbons and lower. Further experiments have shown that addition in the plant growth media of either tridecanoic acid, tridecenoic acid (C13:1 Δ12) or 8-methyl-nonanoic acid resulted in the production of MCL-PHA containing mainly saturated odd-chain, unsaturated odd-chain or branched-chain 3-hydroxyacid monomers, respectively [71]. These results demonstrated that the plant β-oxidation cycle was capable of generating a large spectrum of monomers which can be included in MCL-PHA even from fatty acids which are not present to significant quantities in plants. Furthermore, feeding experiments with these unusual fatty acids demonstrated that all 3-hydroxyacids between 6 and 16 carbons that could be generated by the β-oxidation cycle (via the 3-hydroxyacyl-CoA intermediate) were found into the MCL-PHA. These results supported the concept that monomer composition of PHA could be used as a tool to study the degradation pathway of fatty acids (see Sections 3.3.3 and 3.3.4).

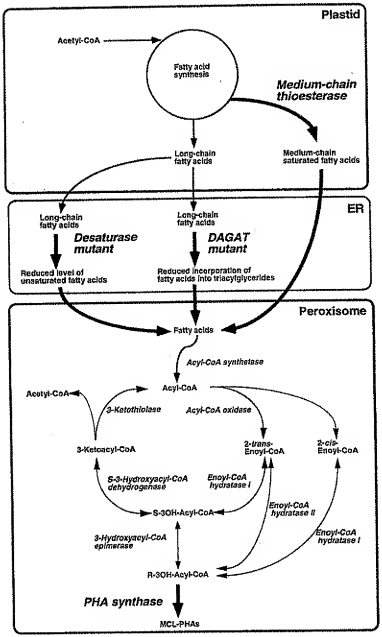

Fig. 6. Modification of plant metabolic pathways for the synthesis of medium-chain-length polyhydroxyalkanoate (MCL-PHA) in the peroxisomes of plants. The pathways created or modified by the expression of transgenes from bacteria (PHA synthase) or plant (medium-chain thioesterase) or the use of mutant genes [diacylglycerol acyltransferase (DAGAT) and fatty acid desaturases] are highlighted in bold.

As an alternative to the addition of external fatty acids, modulation of the monomer composition of MCL-PHA synthesized in peroxisomes was also achieved by modifying the endogenous fatty acid biosynthetic pathway [71]. The first example of this approach was the expression of the peroxisomal PHA synthase in the *A. thaliana* triple mutant *fad3/fad7/fad8*, deficient in the synthesis of the triunsaturated fatty acids [72]. MCL-PHA produced from this mutant was almost completely deficient in all 3-hydroxyacids derived from the degradation of tri-unsaturated fatty acids, including triunsaturated monomers (Table 1) [71]. Since numerous fatty acid desaturases have now been cloned and expressed in transgenic plants to control the number and position of unsaturated bonds in fatty acids, this approach could be extended to further modulate the proportion of a number of 3-hydroxyacid monomers in PHAs.

3.3.3. Studies on futile cycling of fatty acids

One of the first transgenic plants that was created for the synthesis of a novel exotic fatty acids was *B. napus* expressing of the California bay lauroyl-ACP thioesterase [73]. Although expression of the thioesterase under the control of the constitutive CaMV35S promoter led to lauric acid accumulation in seed triacyglycerides, no laurate could be found in leaves, despite the fact that CaMV35S is known to be a strong promoter in this tissue. Eccleston et al. [74] demonstrated that a large fraction of fatty acids synthesized by chloroplast isolated from leaves of these transgenic plants was lauric acid. The observation that the isocitrate lyase activity was increased in these plants supported the hypothesis that in vegetative tissues, the newly synthesized lauric acid was degraded via the β-oxidation cycle instead of accumulating in lipids, thus creating a futile carbon cycle [74]. Analysis of the fate of lauric acid in developing *B. napus* seeds expressing high levels of lauroyl-ACP thioesterase also revealed that a substantial portion of fatty acids were converted to water-soluble compounds [75]. These data, combined with the observed increase in acyl-CoA oxidase activity, indicated that even in developing seeds accumulating lauric acid in triacylglycerols, a substantial portion of lauric acid could be recycled through the β-oxidation cycle.

These studies on lauric-acid producing rapeseed indicated that expression of a thioesterase might be a way of increasing the carbon flux towards β-oxidation and peroxisomal PHA biosynthesis (Fig. 6). This hypothesis was tested in *A. thaliana* by combining the constitutive expression of the peroxisomal PHA synthase with the caproyl-ACP thioesterase from *Cuphea lanceolata* in the plastid [71]. Expression of both enzymes lead to a 7- to 8-fold increase in the amount of MCL-PHA synthesized in plant shoots as compared to transgenics expressing only the PHA synthase (Table 2). Furthermore, the composition of the MCL-PHA in the thioesterase/PHA synthase double transgenic plant was shifted towards saturated 3-hydroxyacid monomers containing 10 carbons and less. This shift is in agreement with an increase in the flux of decanoic acid towards β-oxidation triggered by the expression of the caproyl-ACP thioesterase [71]. Interestingly, constitutive expression of the related lauroyl-ACP thioesterase in *A. thaliana* was shown not to lead to an increase in the genes or enzymes involved in β-oxidation [76]. These results showed that analysis of the quantity and monomer composition of PHA synthesized in the peroxisome could be a more sensitive indicator of the flow of fatty acids towards β-oxidation then the activity of genes or enzymes, both in terms of the nature and relative quantity of fatty acids being re-cycled.

The relation between fatty acid futile cycling and peroxisomal PHA synthesis was further extended to the developing seeds [77]. Synthesis of MCL-PHA has been demonstrated in seeds of

A. thaliana by expressing the peroxisomal PHA synthase gene under the control of the seed-specific napin promoter. In such transgenic plants MCL-PHAs accumulated to 0.006% dwt in mature seeds and the monomer composition was relatively similar to the PHA synthesized in germinating seedlings. Expression of both the PHA synthase and caproyl-ACP thioesterase in the leukoplasts of developing seeds resulted in a nearly 20-fold increase in seed PHA, reaching 0.1% dwt in mature seeds. Furthermore, as found with the expression of these two enzymes in whole plants, co-expression in seeds resulted in a large increase in the proportion of 3-hydroxyacid monomers containing 10 carbons and less in PHA. These data clearly indicate that even though expression of the caproyl-ACP thioesterase in seeds leads to the accumulation of medium-chain fatty acids in triacylglycerols, there is still a proportion of these fatty acids which are channeled towards β-oxidation. This flux towards the β-oxidation cycle is thought to be quite significant, considering that there is only a four-fold difference between the maximal amount of PHA synthesized in germinating seedlings (0.4% dwt), where β-oxidation is thought to be maximal, and the PHA synthesized in the developing seeds expressing the thioesterase (0.1% dwt), where metabolism should be mainly devoted to the synthesis of fatty acid synthesis instead of degradation.

Synthesis of MCL-PHA in the peroxisomes of developing seeds has also demonstrated the presence of an increased cycling of fatty acids towards β-oxidation in plants deficient in the enzyme diacylglycerol acyltransferase (DAGAT) [77]. The *tag*1 mutant of *A. thaliana* was shown to be deficient in DAGAT activity in developing seeds, resulting in a decreased accumulation of triacylglycerols and corresponding increase in diacylglycerols and free fatty acids in mature seeds [78]. It was hypothesized that the imbalance created between that capacity of the plastid to synthesize fatty acids and the capacity of the lipid biosynthetic machinery of the endoplasmic reticulum to include these fatty acids into triacylglycerols might have two basic consequences. These would be that either fatty acid biosynthesis would be reduced (feedback inhibited) in order to match it with triacylglycerol biosynthesis, or that excess fatty acids that cannot be included in triacylglycerols would be channeled towards β-oxidation. Expression of the peroxisomal PHA synthase in the *tag1* mutant resulted in a 10-fold increase in the amount of MCL-PHA accumulating in mature seeds compared to expression of the transgene in wild type plants [77]. Although these results do not address whether fatty acid biosynthesis is decreased in the *tag*1 mutant, they nevertheless clearly indicate that a decrease in triacylglycerol biosynthesis results in a large increase in the flux of fatty acids towards β-oxidation (Fig. 6). Thus, carbon flux to the β-oxidation cycle can be modulated to

Table 2
Composition of medium-chain-length polyhydroxyalkanoate (MCL-PHA) produced in transgenic plant expressing a caproyl-ACP thioesterase

	PHA (mg/g wt)	Monomer composition (mol%)[a]													
		H6	H8	H8:1	H10	H12	H12:1	H12:2	H14	H14:1	H14:2	H14:3	H16	H16:2	H16:3
PHAC3.3	0.1	3.1	13	21	4.7	4.8	2.8	7.5	3.8	4.6	3.7	13	3.0	5.3	10
TP 2.4	0.8	7.2	37	5.8	37	3.6	0.3	1.1	2.4	0.6	0.4	1.4	1.8	0.3	1.5

[a] Monomer composition of MCL-PHA isolated from 40-day-old leaves. Transgenic plant PHAC3.3 expresses only the *P. aeruginosa* PHA synthase in the peroxisome while line TP 2.4 expresses both the *P. aeruginosa* PHA synthase in the peroxisome and the *C. lanceolata* caproyl-ACP thioesterase in the plastid. Adapted from [71]. ACP, acyl carrier protein; PHA, polyhydroxyalkanoate.

a great extent and appears to play an important role in lipid homeostasis in plants even in tissues which are primarily devoted to lipid biosynthesis, such as the developing seeds.

3.3.4. Studies on the degradation of unsaturated and unusual fatty acids

As discussed in Sections 3.3.2 and 3.3.3, changes detected in the monomer composition of peroxisomal MCL-PHAs in plants that are either fed with external fatty acids, are deficient in the synthesis of particular unsaturated fatty acids, or express a medium-chain acyl-ACP thioesterase, clearly reflects both the nature of the fatty acid being degraded and how this fatty acid is degraded by the β-oxidation pathway [71]. It is, thus, possible to use peroxisomal PHA as a tool to elucidate the pathways involved in the degradation of unsaturated and unusual fatty acids. Knowledge on the biochemistry of fatty acid degradation could have an important impact in projects aimed at creating transgenic plants accumulating novel valuable fatty acids, such as ricinoleic acid and vernolic acid [79]. This is because normal germination of a seed from a transgenic oilseed crop accumulating exotic fatty acids may require the presence of additional specialized enzymes required to handle the presence of novel groups on the fatty acids, such as epoxy or hydroxy groups [30]. Furthermore, as revealed by the work with the caproyl-ACP thioesterase, peroxisomal MCL-PHA can be used to measure the extend of the loss of unusual fatty acid in seeds through futile cycling.

The usefulness of using peroxisomal MCL-PHAs to dissect the pathway of fatty acid degradation in plants has recently been demonstrated for the β-oxidation of unsaturated fatty acids [80]. Degradation of fatty acids having *cis*-double bonds on even-numbered carbons requires the presence of auxiliary enzymes in addition to the enzymes of the core β-oxidation cycle. This is because hydration of *cis*-2-enoyl-CoA by the 2-enoyl-CoA hydratase I present in the MFP generates the R-isomer of 3-hydroxyacyl-CoA, which is not a substrate for the S-3-hydroxyacyl-CoA dehydrogenase. Two alternative pathways have been described to degrade these fatty acids [31]. One pathway (referred as the reductase-isomerase pathway) involves the participation of the enzymes 2,4-dienoyl-CoA reductase and Δ^3-Δ^2-enoyl-CoA isomerase, resulting in the conversion of *trans*-2,*cis*-4-dienoyl-CoA to *trans*-2-enyol-CoA, which can be degraded via the core β-oxidation cycle. The second pathway (referred as the epimerase pathway) involves the epimerization of R-3-hydroxyacyl-CoA to the S isomer via a 3-hydroxyacyl-CoA epimerase or the action of two stereo-specific enoyl-CoA hydratases. Whereas degradation of these fatty acids in bacteria and mammalian peroxisomes was shown to mainly involve the reductase-isomerase pathway [81], analysis of the relative activity of the enoyl-CoA hydratase II and 2,4-dienoyl-CoA reductase in plants indicated that degradation occurred mainly, if not exclusively, through the epimerase pathway [70]. The relative contribution of the reductase-isomerase and epimerase pathways could be examined in transgenic plants synthesizing peroxisomal PHA since the degradation of *cis*-10-heptadecenoic or *cis*-10-pentadecenoic acids via these two different pathways results in the introduction of some distinctive 3-hydroxyacid monomers in PHA. For example, degradation of *cis*-10-pentadecenoic acid via the epimerase pathway generates the unsaturated intermediate S-3-hydroxy-*cis*-4-nonenoyl-CoA as well as R-3-hydroxyheptanoyl-CoA while the reductase-isomerase pathway generates the saturated intermediate S-3-hydroxynonanoyl-CoA and S-3-hydroxyheptanoyl-CoA. Analysis of the PHA produced from transgenic plants fed with these different fatty acids revealed that a significant proportion of fatty acid were degraded via the reductase-isomerase pathway in addition of the epimerase pathway [80].

4. Conclusions

A range of PHA have now been synthesized in plants, including the homopolymer PHB, the co-polymer P(HB-HV) as well as a number of MCL-PHAs. The future of the synthesis of PHA in crops as a viable strategy for the production of cheap biodegradable plastics will depend on a number of factors. Perhaps the most important factor will be whether PHA can be produced in high quantity in plants without affecting the overall yield of other plant products, such as oils or proteins. This is important since in contrast to the production of PHA by bacterial fermentation, which is a system primarily designed to produce only PHA, an agricultural production of PHA is likely to be only viable through the recovery of not only PHA, but also all other useful components of the crop. For example, in the case of an oil crop such as *B. napus*, one must be able to recover PHA and the oil, as well as still be able to use the remaining protein-rich material for animal feed. Thus far, we know that PHB can be produced in the seed of rape to 8% dwt without obvious deleterious effects on plant growth [61]. Synthesis of PHA in the plastids of leaves appears to be more limiting since production levels higher then 3–4% dwt leads to chlorosis and growth reduction [57,59]. Although production of PHA in the peroxisome has yielded, so far, only relatively low amount of PHA, this approach has the potential advantage that the carbon used for PHA is not diverted from other anabolic pathways involved in the synthesis of essential compounds, such as fatty acids or amino acids, but is rather derived from catabolic pathways. It is thus likely that synthesis of high amount of PHA in peroxisome may have less of a metabolic penalty then synthesis of PHA in the plastid.

Beyond its direct impact in plant biotechnology as a biopolymer production system, synthesis of PHA in plants can also be exploited as a novel tool to study metabolic pathways. Because PHA represent a largely un-recyclable carbon sink in plants, it can be used to study the response of plants to changes in carbon flux to various pathways, either at the genetic, enzymatic, or metabolite levels. As well demonstrated by the observation of higher accumulation of PHA in cells of the leaf vascular bundle compared to mesophyll cells in corn, PHA can also reveal hidden differences in the quantity and/or availability of metabolites in different cells and tissues. So far, PHA as an analytical tool has been mostly exploited to study fatty acid degradation in the peroxisome. In this area, as well as others, PHA is likely to continue to contribute significantly to our understanding of plant biochemistry.

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Posttranslational modification

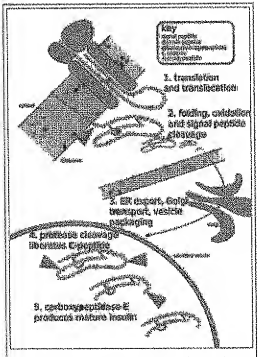
From Wikipedia, the free encyclopedia
(Redirected from Post-translational modification)

Posttranslational modification (PTM) is the chemical modification of a protein after its translation. It is one of the later steps in protein biosynthesis for many proteins.

A protein (also called a polypeptide) is a chain of amino acids. During protein synthesis, 20 different amino acids can be incorporated in proteins. After translation, the posttranslational modification of amino acids extends the range of functions of the protein by attaching to it other biochemical functional groups such as acetate, phosphate, various lipids and carbohydrates, by changing the chemical nature of an amino acid (e.g. citrullination) or by making structural changes, like the formation of disulfide bridges.

Also, enzymes may remove amino acids from the amino end of the protein, or cut the peptide chain in the middle. For instance, the peptide hormone insulin is cut twice after disulfide bonds are formed, and a propeptide is removed from the middle of the chain; the resulting protein consists of two polypeptide chains connected by disulfide bonds.

Other modifications, like phosphorylation, are part of common mechanisms for controlling the behavior of a protein, for instance activating or inactivating an enzyme.



The bottom of this diagram shows the modification of primary structure of insulin, as described.

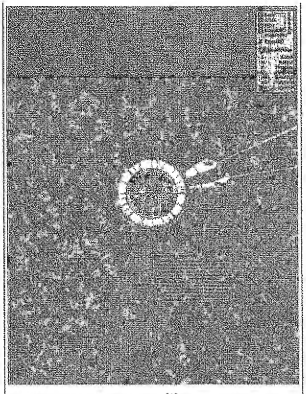
Contents

PTMs involving addition of functional groups

PTMs involving addition include:

- acylation
  - acetylation, the addition of an acetyl group, usually at the N-terminus of the protein

- alkylation, the addition of an alkyl group (e.g. methyl, ethyl)
  - methylation the addition of a methyl group, usually at lysine or arginine residues. (This is a type of alkylation.)
  - demethylation
- amidation at C-terminus
- biotinylation, acylation of conserved lysine residues with a biotin appendage
- formylation
- gamma-carboxylation dependent on Vitamin K[2]
- glutamylation, covalent linkage of glutamic acid residues to tubulin and some other proteins.[3] (See tubulin polyglutamylase)
- glycosylation, the addition of a glycosyl group to either asparagine, hydroxylysine, serine, or threonine, resulting in a glycoprotein. Distinct from glycation, which is regarded as a nonenzymatic attachment of sugars.
- glycylation, covalent linkage of one to more than 40 glycine residues to the tubulin C-terminal tail
- heme moiety may be covalently attached
- hydroxylation
- iodination (e.g. of thyroid hormones)
- isoprenylation, the addition of an isoprenoid group (e.g. farnesol and geranylgeraniol)
- lipoylation, attachment of a lipoate functionality
  - prenylation
  - GPI anchor formation
    - myristoylation
    - farnesylation
    - geranylgeranylation
- nucleotides or derivatives thereof may be covalently attached
  - ADP-ribosylation
  - flavin attachment
- oxidation
- palmitoylation
- pegylation
- phosphatidylinositol may be covalently attached
- phosphopantetheinylation, the addition of a 4'-phosphopantetheinyl moiety from coenzyme A, as in fatty acid, polyketide, non-ribosomal peptide and leucine biosynthesis
- phosphorylation, the addition of a phosphate group, usually to serine, tyrosine, threonine or histidine
- polysialylation, addition of polysialic acid, PSA to NCAM
- pyroglutamate formation
- racemization of proline by prolyl isomerase
- tRNA-mediation addition of amino acids such as arginylation
- sulfation, the addition of a sulfate group to a tyrosine.
- selenoylation (co-translational incorporation of selenium in selenoproteins)



The genetic code diagram[1] showing the amino acid residues as target of modification.

- sulfation

PTMs involving addition of other proteins or peptides

- ISGylation, the covalent linkage to the ISG15 protein (Interferon-Stimulated Gene 15)[4]
- SUMOylation, the covalent linkage to the SUMO protein (Small Ubiquitin-related MOdifier)[5]
- ubiquitination, the covalent linkage to the protein ubiquitin.

PTMs involving changing the chemical nature of amino acids

- citrullination, or **deimination** the conversion of arginine to citrulline
- deamidation, the conversion of glutamine to glutamic acid or asparagine to aspartic acid

PTMs involving structural changes

- disulfide bridges, the covalent linkage of two cysteine amino acids
- proteolytic cleavage, cleavage of a protein at a peptide bond

Case examples

- cleavage and formation of disulfide bridges during the production of insulin
- PTM of histones as regulation of transcription: RNA polymerase control by chromatin structure
- PTM of RNA polymerase II as regulation of transcription: RNA polymerase II

External links

- deltaMasses: Differential PTM Detection after mass spectrometry
- AutoMotif Server: A Computational Protocol for Identification of Post-Translational Modifications in Protein Sequences
- Functional analyses for site-specific phosphorylation of a target protein in cells

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Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic

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INTRODUCTION TO POLY(3-HYDROXYALKANOATES)

Storage Material

Poly(3-hydroxyalkanoates) (PHAs) are structurally simple macromolecules synthesized by many gram-positive and gram-negative bacteria. PHAs are accumulated as discrete granules to levels as high as 90% of the cell dry weight and are generally believed to play a role as sink for carbon and reducing equivalents. When nutrient supplies are imbalanced, it is advantageous for bacteria to store excess nutrients intracellularly, especially as their general fitness is not affected. By polymerizing soluble intermediates into insoluble molecules, the cell does not undergo alterations of its osmotic state and leakage of these valuable compounds out of the cell is prevented. Consequently, the nutrient stores will remain available at a relatively low maintenance cost and with a secured return on investment (36, 182, 239, 240, 286).

Once PHAs are extracted from the bacterial cell, however, these molecules show material properties that are similar to some common plastics such as polypropylene (20). The bacterial origin of the PHAs make these polyesters a natural material, and, indeed, many microorganisms have evolved the ability to degrade these macromolecules. Besides being biodegradable, PHAs are recyclable like the petrochemical thermoplasts. This review summarizes the chemical and physical properties of PHAs and the biochemical and genetic studies of the pathways involved in PHA metabolism. Within this framework, the scientific advances that have been made with the available *phe* genes for economic PHA production processes will be described.

Chemical Structure

The many different PHAs that have been identified to date are primarily linear, head-to-tail polyesters composed of 3-hydroxy fatty acid monomers. In these polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Fig. 1). In all PHAs that have been characterized so far, the hydroxyl-substituted carbon atom is of the *R* configuration, except in some special cases where there is no chirality. At the same C-3 or β position, an alkyl group which can vary from methyl to tridecyl is positioned. However, this alkyl side chain is not necessarily saturated: aromatic, unsaturated, halogenated, epoxidized, and branched monomers have been reported as well (1, 25, 32, 44, 58–60, 85, 125, 126, 135, 247). Specialized, unnatural monomers such as 4-cyanophenylvalerate have been incorporated to obtain new polymers with special properties (124). As well as the variation in the alkyl substituent, the position of the hydroxyl group is somewhat variable, and 4-, 5- and 6-hydroxy acids have been incorporated (51, 131, 277–279). Substituents in the side chains of PHAs can be modified chemically, for instance by cross-linking of unsaturated bonds (39, 67, 68). This variation in the length and composition of the side chains and the ability to modify their reactive substituents is the basis for the diversity of the PHA polymer family and their vast array of potential applications that are described below.

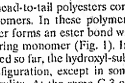

FIG. 1. Chemical structure of PHAs. PHAs are generally composed of (*R*)-β-hydroxy fatty acids, where the pendant group (R) varies from methyl (C_1) to tridecyl (C_{13}). Other fatty acids that have been incorporated have the hydroxy group at the γ, δ, or ε position, while the pendant group may be saturated or unsaturated or contain substituents. The best-known PHAs are P(3HB) (R = methyl), P(3HB-3HV) (R = methyl or ethyl), and P(3HO-3HH) (R = pentyl or propyl).

Historically, poly(3-hydroxybutyrate) [P(3HB)] has been studied most extensively and has triggered the commercial interest in this class of polymers. P(3HB) is the most common type of PHA, and the ability of bacteria to accumulate P(3HB) is often used as a taxonomic characteristic. Copolymers of P(3HB) can be formed by cofeeding of substrates and may result in the formation of polymers containing 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers. Together, polymers containing such monomers form a class of PHAs typically referred to as short-side-chain PHAs (ssc-PHAs). In contrast, medium-side-chain PHAs (msc-PHAs) are composed of C_6 to C_{16} 3-hydroxy fatty acids. These PHAs are synthesized from fatty acids or other aliphatic carbon sources, and, typically, the composition of the resulting PHA depends on the growth substrate used (17, 105, 135). msc-PHAs are also synthesized from carbohydrates, but the composition of these PHAs is not related to the carbon source (84, 102, 270). The vast majority of microbes synthesize either ssc-PHAs containing primarily 3HB units or msc-PHAs containing 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as the major monomers (6, 142, 249, 252).

Physical Characteristics

The molecular mass of PHAs varies per PHA producer but is generally on the order of 50,000 to 1,000,000 Da. Although aliphatic polyesters have been studied extensively since the 1920s, their properties were not remarkable and did not initiate a great commercial interest at that time. This was primarily due to the use of relatively impure substrates at the time, which limited the molecular masses of these polymers to 20,000 to 30,000 Da (159). Bacterially produced P(3HB) and other PHAs, however, have a sufficiently high molecular mass to have polymer characteristics that are similar to conventional plastics such as polypropylene (Table 1).

Within the cell, P(3HB) exists in a fluid, amorphous state. However, after extraction from the cell with organic solvents, P(3HB) becomes highly crystalline (43) and in this state is a stiff but brittle material. Because of its brittleness, P(3HB) is not very stress resistant. Also, the relatively high melting temperature of P(3HB) (around 170°C) is close to the temperature where this polymer decomposes thermally and thus limits the

TABLE 1. Properties of PHAs and polypropylene[a]

Parameter	Value for[b]:				
	P(3HB)	P(3HB-3HV)	P(3HB-4HB)	P(3HO-3HH)	PP
T_m (°C)[c]	177	145	150	61	176
T_g (°C)[d]	2	−1	−7	−36	−10
Crystallinity (%)	70	56	45	30	60
Extension to break (%)	5	50	444	300	400

[a] Data from reference 42.
[b] P(3HB) is poly(3-hydroxybutyrate), P(3HB-3HV) is poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing 20% 3HV, P(3HB-4HB) is poly(3-hydroxybutyrate-co-4-hydroxybutyrate) containing 16% 4HB, P(3HO-3HH) is poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) containing 11% 3HH, and PP is polypropylene.
[c] T_m is melting temperature.
[d] T_g is glass transition temperature.

ability to process the homopolymer. Initial biotechnological developments were therefore aimed at making PHAs that were easier to process. The incorporation of 3HV into the P(3HB) resulted in a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-3HV)] copolymer that is less stiff and brittle than P(3HB), that can be used to prepare films with excellent water and gas barrier properties reminiscent of polypropylene, and that can be processed at a lower temperature while retaining most of the other excellent mechanical properties of P(3HB) (159). In contrast to P(3HB) and P(3HB-3HV), msc-PHAs have a much lower level of crystallinity and are more elastic (73, 208). These msc-PHAs potentially have a different range of applications from the ssc-PHAs.

Biological Considerations

The diversity of different monomers that can be incorporated into PHAs, combined with a biological polymerization system that generates high-molecular weight materials, has resulted in a situation where an enormous range of new polymers are potentially available. The advent of genetic engineering combined with modern molecular microbiology now provides us with the exceptional framework for studying plastic properties as a function of genetic and metabolic blueprints. In fact, it presents an enormous challenge to our scientific discipline to fully explore this biology to ensure that environmentally friendly polyesters are available for generations to come.

Biodegradability. Besides the typical polymeric properties described above, an important characteristic of PHAs is their biodegradability. In nature, a vast consortium of microorganisms is able to degrade PHAs by using secreted PHA hydrolases and PHA depolymerases (for a review of the microbiology and molecular genetics of PHA degradation, see reference 111). The activities of these enzymes may vary and depend on the composition of the polymer, its physical form (amorphous or crystalline), the dimensions of the sample, and, importantly, the environmental conditions. The degradation rate of a piece of P(3HB) is typically on the order of a few months (in anaerobic sewage [Fig. 2]) to years (in seawater) (111, 167–169).

Renewable nature. As important as the biological characteristics and biodegradability of PHAs is the fact that their production is based on renewable resources. Fermentative production of PHAs is based on agricultural products such as sugars and fatty acids as carbon and energy sources. These agricultural feedstocks are derived from CO_2 and water, and after their conversion to biodegradable PHA, the breakdown

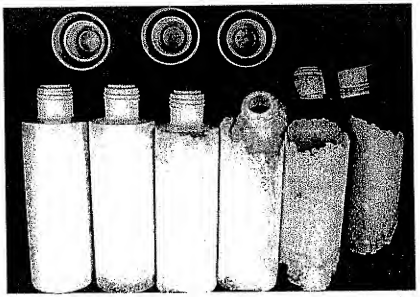

FIG. 2. Degradation of P(3HB-3HV) in aerobic sewage sludge. Bottles made of P(3HB-3HV) were incubated during the summer (average temperature, 20°C) in aerobic sewage sludge. The progress of degradation is demonstrated with bottles that have been subjected to this treatment for 0, 2, 4, 6, 8, and 10 weeks (from left to right). Photograph courtesy of Dieter Jendrossek, Georg-August-Universität, Göttingen, Germany.

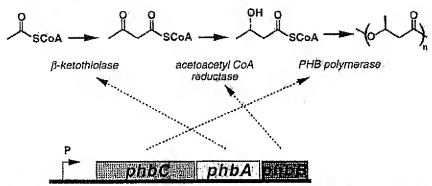

FIG. 3. Biosynthetic pathway for P(3HB). P(3HB) is synthesized in a three-step pathway by the successive action of β-ketoacyl-CoA thiolase (PhbA), acetoacetyl-CoA reductase (PhbB), and P(3HB) polymerase (PhbC). The three enzymes are encoded by the genes of the *phbCAB* operon. A promoter upstream of *phbC* transcribes the complete operon.

products are again CO_2 and water. Thus, while for some applications the biodegradability is critical, PHAs receive general attention because they are based on renewable compounds instead of on our diminishing fossil fuel stockpiles (293).

Applications

PHAs are natural thermoplastic polyesters, and hence the majority of their applications are as replacements for petrochemical polymers currently in use for packaging and coating applications. The extensive range of physical properties of the PHA family of polymers and the broadened performance obtainable by compounding and blending provide a correspondingly broad range of potential end-use applications, as described in numerous patents.

Initial efforts focused on molding applications, in particular for consumer packaging items such as bottles, cosmetic containers, pens, and golf tees (9, 10, 287). U.S. patents 4,826,493 and 4,880,592 describe the manufacture of P(3HB) and P(3HB-3HV) films and their use as diaper backsheet (163, 164). These films can also be used to make laminates with other polymers such as polyvinyl alcohol (91). Diaper backsheet materials and other materials for manufacturing biodegradable or compostable personal hygiene articles from P(3HB) copolymers other than P(3HB-3HV) have been described (180, 181, 241). PHAs have also been processed into fibers which then were used to construct materials such as nonwoven fabrics (248). P(3HB) and P(3HB-3HV) have been described as hot-melt adhesives (118). PHAs with longer-side-chain hydroxyacids have been used in pressure-sensitive adhesive formulations (229). PHAs can also be used to replace petrochemical polymers in toner and developer compositions (65) or as ion-conducting polymers (221, 222). PHAs can be used as a latex, for instance for paper-coating applications (160), or can be used to produce dairy cream substitutes (298) or flavor delivery agents in foods (299).

In addition to its range of material properties and resulting applications, PHAs promise to be a new source of small molecules. PHA can be hydrolyzed chemically, and the monomers can be converted to commercially attractive molecules such as are β-hydroxy acids, 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids, and β-hydroxyacid esters (293). The last class of chemicals is currently receiving attention because of potential applications as biodegradable solvents.

PHA BIOSYNTHESIS IN NATURAL ISOLATES

Since 1987, the extensive body of information on P(3HB) metabolism, biochemistry, and physiology has been enriched by molecular genetic studies. Numerous genes encoding enzymes involved in PHA formation and degradation have been cloned and characterized from a variety of microorganisms. From these studies, it is becoming clear that nature has evolved several different pathways for PHA formation, each optimized for the ecological niche of the PHA-producing microorganism. Genetic studies have, furthermore, given insights into the regulation of PHA formation with respect to growth conditions. The cellular physiology of the cell and the important role of central metabolism have become apparent by studying PHA mutants with modifications in genes other than the *phb* genes. Not only do such studies provide a fundamental insight into microbial physiology, but also they provide the keys for designing and engineering recombinant organisms for PHA production. This section deals with the molecular details of the PHA enzymes and corresponding genes and how their activities blend with cellular metabolism to synthesize PHA only at times where their synthesis is useful.

Of all the PHAs, P(3HB) is the most extensively characterized polymer, mainly because it was the first to be discovered, in 1926 by Lemoigne at the Institute Pasteur (152). The P(3HB) biosynthetic pathway consists of three enzymatic reactions catalyzed by three distinct enzymes (Fig. 3). The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by β-ketoacyl-CoA thiolase (encoded by *phbA*). The second reaction is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by *phbB*). Lastly, the (*R*)-3-hydroxybutyryl-CoA monomers are polymerized into poly(3-hydroxybutyrate) by P(3HB) polymerase (encoded by *phbC*). Although P(3HB) accumulation is a widely distributed prokaryotic phenotype, the biochemical investigations into the enzymatic mechanisms of β-ketoacyl-CoA thiolase, acetoacetyl-CoA reductase, and P(3HB) polymerase have focused on only two of the natural producers, *Zoogloea ramigera* and *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*).

Essential Genes for PHA Formation

The first *phb* gene to be isolated was from *Z. ramigera* (190), an interesting bacterium for biopolymer engineering since it

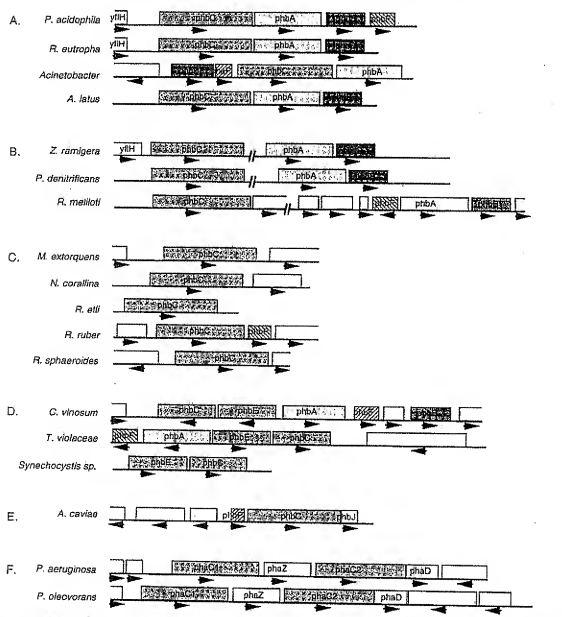

FIG. 4. *pha* and *phb* operons. The loci encoding the genes for PHA formation have been characterized from 18 different species. Genes specifying enzymes for sse-PHA formation are designated *phb*, and those specifying enzymes for nsse-PHA formation are designated *pha*. Not all pathways have completely been elucidated in these strains. The emerging picture is that *pha* and *phb* genes are not necessarily clustered and that the gene organization varies from species to species. Other genes possibly related to PHA metabolism may be linked to the essential *pha* and *phb* genes. (A) Complete *phbCAB* operons. (B) Interrupted *phb* loci. (C) Incomplete *phb* loci. (D) *phb* loci from organisms that encode two subunit P(3HB) polymerases. (E) The *phbCJ* locus of *A. caviae* involved in P(3HB-3HH) formation. (F) *pha* loci for nsse-PHA formation in *Pseudomonas*.

produces both P(3HB) and extracellular polysaccharide (50). By using anti-thiolase antibodies the *phbA* gene was detected in *Escherichia coli* carrying a *Z. ramigera* gene library and was subsequently cloned (190). It was found that *phbA* and *phbB* form an operon, while *phbC* is located elsewhere on the chromosome of *Z. ramigera* (191). The cloning of *phbA* and *phbB* facilitated the purification of the encoded ketoacyl-CoA thiolase and acetoacetyl-CoA reductase for kinetic and mechanistic characterization of these enzymes as described in later sections.

Since the original discovery of these *phb* genes, many genes encoding enzymes from the PHA pathway have been cloned from different organisms (Fig. 4). Given the diversity of P(3HB) biosynthetic pathways, it is not surprising that the *pha* loci have diverged considerably. In *Acinetobacter* spp., *Alcaligenes latus*, *Pseudomonas acidophila*, and *R. eutropha*, the *phbCAB* genes are in tandem on the chromosome although not necessarily in the same order (108, 192, 193, 232, 274). In *Paracoccus denitrificans*, *Rhizobium meliloti*, and *Z. ramigera*, the *phbAB* and *phbC* loci are unlinked (141, 191, 271, 273, 297). PHA polymerase in *Chromatium vinosum*, *Thiocystis violacea*, and *Synechocystis* is a two-subunit enzyme encoded by

the *phbE* and *phbC* genes. In these organisms, *phbAB* and *phbEC* are in one locus but divergently oriented (87, 154, 155). The *phb* loci in *C. vinosum*, *P. acidophila*, *R. eutropha*, *Rhizobium meliloti*, and *T. violacea* all have an additional gene, *phbF*, that has a hitherto unknown function in PHA metabolism (202), while part of a gene encoding a protein homologous to the hypothetical *E. coli* protein YfiH is located upstream of the *P. acidophila*, *R. eutropha*, and *Z. ramigera* P(3HB) polymerase genes. In *Methylobacterium extorquens*, *Nocardia corallina*, *Rhizobium etli*, *Rhodococcus ruber*, and *Rhodobacter sphaeroides*, only the PHB polymerase-encoding gene has been identified thus far (23, 78, 109, 195, 280). The PHA polymerase gene in *Aeromonas caviae* is flanked by a unique PHA biosynthetic enzyme encoded by *phaJ*, which is discussed in further detail below (61). In mcl-PHA-producing *P. oleovorans* and *P. aeruginosa*, the *pha* loci contain two *phaC* genes (107, 269) separated by *phaZ*, which encodes an intracellular PHA depolymerase (107). The two PHA polymerases are 50 to 60% identical in their primary structure and appear to have a very similar substrate specificity (102, 107).

Figure 4 provides grounds for some speculation on the evolution of PHA formation. When the first PHA-forming bacteria used this pathway, the purpose of the pathway was probably different from synthesis of a storage material (see also below). PHA formation was most probably a minor metabolic pathway in these organisms, perhaps resulting only from a side reaction. When PHA formation became beneficial for the microbe, evolution selected for improved PHA-accumulating strains under conditions of which we are unfortunately not aware. Knowledge of such conditions would be extremely helpful in the current efforts to optimize PHA production that employ recombinant PHA producers and are described in later sections. Over the course of evolution, *phaC* was sometimes combined with genes that supply monomer, such as *phbAB* or *phaJ*, or with genes involved in other aspects of PHA metabolism, such as *phaZ*. The selective pressures active at the time resulted in the clustering of *pha* genes in an operon in some organisms (as in *P. acidophila*, *R. eutropha*, *Acinetobacter*, *Alcaligenes latus*, and *Aeromonas caviae*) or as separate transcriptional units in others (as in *Z. ramigera*, *P. denitrificans*, *Rhizobium meliloti*, *C. vinosum*, *T. violacea*, *P. oleovorans*, *P. putida*, and perhaps other microorganisms for which no thiolase and reductase genes have been identified yet). A second evolutionary force must have worked on the *pha* genes since some but not all of these diversely structured loci contain *phbF* and *phbF* genes or homologs of *yfiH*. Whether the ancestral PHA polymerase was encoded by one (*phaC*) or two (*phaEC*) open reading frames is an open question. Since the two-subunit polymerase systems in *C. vinosum* and *T. violaceae* do have neighboring thiolase and reductase genes whereas *phaEC* in *Synechocystis* does not, fusion of *phaEC* or splicing of *phaC* may have preceded the rearrangements in the *pha* loci.

Although *B. megaterium* was the first strain from which P(3HB) was isolated and identified, its biosynthetic machinery has not yet been characterized. The recently isolated *B. megaterium* mutants impaired in P(3HB) formation (55) should allow the cloning and characterization of the *phb* genes from this historically relevant P(3HB) producer.

The Three-Step ssc-PHA Biosynthetic Pathway

β-Ketoacyl-CoA thiolase. β-Ketoacyl-CoA thiolase catalyzes the first step in P(3HB) formation. The P(3HB) biosynthetic thiolase (acetyl-CoA:acetyl-CoA-acetyl transferase; EC 2.3.1.9) is a member of a family of enzymes involved in the thiolytic cleavage of substrates into acyl-CoA plus acetyl-CoA. These β-ketoacyl-CoA thiolases are found throughout nature from higher eukaryotes to yeasts to prokaryotes and are divided into two groups based on their substrate specificity. The first group consists of thiolases with a broad specificity for β-ketoacyl-CoAs ranging from C_4 to C_{16}. This class of enzymes is involved mainly in the degradation of fatty acids and is located in the cytoplasm of prokaryotes and in the mitochondria and peroxisomes of mammalian and plant cells. The second class of β-ketoacyl-CoA thiolases is considered biosynthetic and has a narrow range of chain length specificity, from C_3 to C_5. Throughout nature, these biosynthetic thiolases are specialized for a variety of roles such as ketone body formation, steroid and isoprenoid biosynthesis, and P(3HB) synthesis. The thiolase involved in P(3HB) formation is a biosynthetic thiolase with specificity primarily for acetoacetyl-CoA (166).

R. eutropha contains two β-ketothiolases (enzyme A and enzyme B) that are able to act in the biosynthetic pathway to P(3HB) synthesis. The major difference between these two enzymes is their substrate specificity. Enzyme A is a homotetramer of 44-kDa subunits and converts acetoacetyl-CoA and 3-ketopentanoyl-CoA (but only at 3% relative activity in comparison to acetoacetyl-CoA). In contrast, enzyme B, a homotetramer of 46-kDa subunits, has a broader substrate specificity and cleaves acetoacetyl-CoA as well as 3-ketopentanoyl-CoA, 3-ketohexanoyl-CoA, 3-ketoheptanoyl-CoA, 3-ketooctanoyl-CoA, and 3-ketodecanoyl-CoA (30, 17, 19, 10, and 12% activity relative to acetoacetyl-CoA, respectively). Originally it was thought that the major role of enzyme B is in fatty acid degradation while the primary role of enzyme A (PhbA) is in the biosynthesis of P(3HB) (81). Recently, however, it has been shown that enzyme B is the primary source of the 3HV monomer for P(3HB-3HV) formation (244).

The enzymatic mechanism of PhbA consists of two half-reactions that result in the condensation of two acetyl-CoA molecules into acetoacetyl-CoA. In the first half-reaction, an active-site cysteine attacks an acetyl-*S*-CoA molecule to form an acetyl-*S*-enzyme intermediate. In the second half-reaction, a second cysteine deprotonates another acetyl-CoA, resulting in an activated acetyl-CoA intermediate that is able to attack the acetyl-*S*-enzyme intermediate and form acetoacetyl-CoA (165). The involvement of a cysteine(s) in the active site of the P(3HB) thiolase was first hypothesized in 1953 because the thiolase was inhibited by sulfhydryl-blocking agents (156). In the late 1980s, the roles of cysteines in the active site of the P(3HB) thiolase were definitively determined, after the thiolase gene from *Z. ramigera* had been cloned and the enzyme had been overproduced and purified. The cysteine involved in the acetyl-*S*-enzyme intermediate was identified as Cys89 by peptide sequencing of the radioactive peptide after tryptic digestion of radiolabeled enzyme with [^{14}C]iodoacetamide or [^{14}C]acetyl-CoA (35, 267). A C89S thiolase mutant was also constructed and determined to be severely affected in catalysis but not substrate affinity (165, 267). The second cysteine in the active site of P(3HB) thiolase was determined by using affinity-labeled inactivators such as bromoacetyl-*S*-pantetheine-11-pivalate. By using this inhibitor, Cys378 was identified as a potential residue for the second active-site cysteine that deprotonates the second acetyl-CoA molecule (34, 186) and the C378G mutant was virtually inactive (165, 186). So far, all P(3HB) thiolases contain these two active-site cysteines, and it is believed that all the P(3HB) thiolases use the same enzymatic mechanism to condense acetyl-CoA with either acetyl-CoA or acyl-CoA.

Acetoacetyl-CoA reductase. Acetoacetyl-CoA reductase is an (*R*)-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.36) and catalyzes the second step in the P(3HB) biosynthetic pathway

TABLE 2. Kinetic characteristics of P(3HB) biosynthetic enzymes

Enzyme and species	K_m (mM)	Substrate	Product	Reference
Thiolase (condensation)				
Z. ramigera	0.33	Acetyl-CoA	Acetoacetyl-CoA	35
Thiolase (thiolysis)				
Z. ramigera	0.024	Acetoacetyl-CoA	Acetyl-CoA	35
	0.46	Acetoacetyl-pantheteine	Acetyl-CoA + acetyl-pantheteine	35
	0.073	Acetoacetyl-pantheteine-11-pivalate	Acetyl-CoA + acetyl-pantheteine-11-pivalate	35
	(50%)[a]	3-Ketovaleryl-CoA	Acetyl-CoA + propionyl-CoA	166
R. eutropha	0.044	Acetoacetyl-CoA	Acetyl-CoA	252
	(3%)[b]	3-Ketovaleryl-CoA	Acetyl-CoA + propionyl-CoA	252
	(0%)[b]	3-Ketohexanoyl-CoA	Acetyl-CoA + butanoyl-CoA	252
NADPH-dependent reductase				
Z. ramigera	0.002	Acetoacetyl-CoA	3-Hydroxybutyryl-CoA	198
	0.002	3-Ketovaleryl-CoA	3-Hydroxyvaleryl-CoA	198
	0.010	3-Ketohexanoyl-CoA	3-Hydroxyhexanoyl-CoA	198
	0.99	Acetoacetyl-pantheteine-11-pivalate	3-Hydroxybutyryl-pantheteine-11-pivalate	198
R. eutropha	0.005	Acetoacetyl-CoA	3-Hydroxybutyryl-CoA	252
	(18%)[b]	3-Ketovaleryl-CoA	3-Hydroxyvaleryl-CoA	252
	(3.6%)[b]	3-Ketohexanoyl-CoA	3-Hydroxyhexanoyl-CoA	252
P(3HB) polymerase				
R. eutropha	0.72	3-Hydroxybutyryl-CoA	P(3HB)	252
	1.63	3-Hydroxyvaleryl-CoA	PHV	252
	ND[c]	3-Hydroxybutyryl-pantheteine-11-pivalate	None	69

[a] V_{max} with respect to acetoacetyl-CoA.
[b] Relative activity with respect to acetoacetyl-CoA and 3-hydroxybutyryl-CoA.
[c] ND, not determined.

by converting acetoacetyl-CoA into 3-hydroxybutyryl-CoA. The acetoacetyl-CoA reductase from *Z. ramigera* is a homotetramer of 25-kDa subunits and has been classified as an NADPH-dependent reductase (62, 198, 231). Although both NADPH- and NADH-dependent acetoacetyl-CoA reductase activities have been observed in cell extracts of *R. eutropha*, only the former is involved in P(3HB) synthesis (82). The only known NADH-dependent acetoacetyl-CoA reductase involved in P(3HB) formation to date was found in *C. vinosum* (155). Although the *phbB* gene product from *Paracoccus denitrificans* was initially ascribed to be NADH dependent (297), subsequent overexpression of this enzyme and characterization proved this reductase to be active only with NADPH (29).

The enzymatic reactions involved in P(3HB) synthesis have been extensively analyzed by biochemical techniques and provide clues about the regulation of this pathway. The preferred reaction for the thiolase is thiolytic cleavage, which occurs in the direction opposite to the P(3HB) biosynthetic pathway. However, under P(3HB)-accumulating conditions the enzyme acts against its thermodynamically favored direction when the activities of acetoacetyl-CoA reductase and P(3HB) polymerase pull the condensation reaction (reviewed in reference 166). The availability of reducing equivalents in the form of NADPH is therefore considered to be the driving force for P(3HB) formation.

In the P(3HB) biosynthetic pathway, the reactions catalyzed by thiolase and reductase provide the monomer for PHA polymerization. The kinetic characteristics and substrate specificities of these two enzymes are therefore crucial in determining the range of products that can be expected to be synthesized in a thiolase, reductase, polymerase pathway, as depicted in Fig. 3. Table 2 shows a compilation of the kinetic characteristics of the best-studied thiolase and reductase enzymes, which provides insights in the use of these enzymes for the formation of P(3HB) copolymers. The concept of dividing PHA formation into monomer supply pathways and polymerization is important since in later sections it will be shown that monomers are not necessarily supplied by dedicated pathways. Some of the strategies currently used in fermentative production processes and also the new developments in metabolic engineering provide examples of the incorporation of monomers that are not supplied by thiolase and/or reductase mediated reactions.

P(3HB) polymerase. P(3HB) polymerase is the third enzyme in the biosynthetic pathway for P(3HB) production. The first *phbC* nucleotide sequence to be reported was from *R. eutropha*. This gene was isolated by complementation of *R. eutropha* P(3HB)-negative mutants (192), and the promoter that drives the expression of *phbC* (235) and the other genes in the *phb* operon (192, 193) was mapped. Expression of these three genes in *E. coli* resulted in the accumulation of P(3HB) up to levels exceeding 50% of the cell dry weight (192, 236, 245).

P(3HB) polymerase is just one member of the family of PHA polymerases. All of the polymerases have molecular masses of around 63,000 Da, except for the polymerases from *C. vinosum* (153), *T. violacea* (154), and *Synechococcus* spp. (87, 114), which are composed of two subunits with molecular masses of 40 and 45 kDa. Interestingly, there are only 15 fully conserved residues among the 26 known PHA polymerases, many of which lead only to sce-PHA formation (Fig. 5). This is remarkable, since these 15 residues represent on average less than 3% of the total number of amino acids in these enzymes. Since PHA polymerase is found in both soluble (hydrophilic) and granule-bound (hydrophobic) states, it may be that evolution has selected for enzymes that are catalytically efficient while presenting few problems related to undesirable "protein-hydrophobic-surface" interactions. The broad variety of PHA-producing microbes would represent a vast spectrum of intracellular conditions to which these enzymes would have to

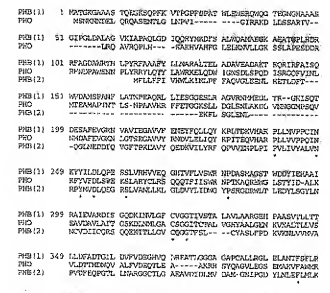

FIG. 5. Sequence similarity of representatives of three types of PHA polymerases. *R. eutropha* ssc-PHA polymerase (PHB1), *P. oleovorans* msc-PHA polymerase (PHO), and the PhbC subunit of the two-subunit polymerase from *Spirulinaplatt* sp. (PHB2) were aligned by using the program of Higgins (MacDNASIS; IntelliGenetics, Mountain View, Calif.). Residues conserved in all PHA polymerases identified to date are marked by an asterisk.

be adapted. This could explain the low level of overall conserved sequence identity between the different PHA polymerases.

Early biochemical studies of PHB polymerase were hampered by the low activity of the protein purified from the natural PHB producers. These studies, however, indicated that the enzyme exists in both soluble and granule-bound forms (64, 83). It was proposed that two cysteine residues might be involved in catalysis, with one cysteine holding the growing PHA chain while the other cysteine holds the incoming monomer (72). To test this theory, two cysteines (Cys319 and Cys459) in the *R. eutropha* P(3HB) polymerase were mutated (70). Cys319 is conserved in all the synthases isolated to date (250), while Cys459 is conserved between only the *R. eutropha* and the *P. oleovorans* PHA polymerases. Cys319 was shown to be an active-site residue, because serine and alanine mutations rendered the enzyme inactive. In contrast, when the second cysteine (Cys459) was mutated to a serine, the enzyme retained 90% of the wild-type activity (70). By using the tritiated trimer $(3HB)_3$-CoA, it was shown that the P(3HB) polymer is covalently bound to the P(3HB) polymerase through Cys319 (296).

To explain the ability of the enzyme to form ester bonds with only one cysteine residue, a second thiol was proposed to exist via posttranslational modification. Phosphopantetheine was proposed as a potential posttranslational modification moiety for P(3HB) polymerase (70). A phosphopantetheine posttranslational modification has been found in acyl-carrier protein and enzymes in enterobactin biosynthesis (110). By using a P(3HB) polymerase overexpression system, it was shown that the PhbC enzyme is radioactively labeled when β-[^{3}H]alanine, a precursor of phosphopantetheine, is supplied to the culture. The most likely residue to be modified by phosphopantetheinylation is Ser260 (70), a residue conserved in all *phaC* genes characterized to date (Fig. 5) and part of a region that resembles similar sites in pantothenylated enzymes (70).

Given the function of the polymerases in forming ester bonds, it is not surprising to find the active-site cysteine residue of these enzymes in a lipase box; Gly-X-Cys^{319}-X-Gly-Gly. The active site of a lipase generally consists of a nucleophile, either cysteine or serine, whose reactivity is enhanced by an aspartate residue and a histidine residue (16, 194, 295). Together, these three residues form a catalytic triad. Candidates for these aspartate and histidine residues are conserved in the polymerases, namely, aspartate residues at positions 351, 428, and 480 and histidine residues at positions 481 and 508 (Fig. 5). Given that PHA polymerase may have two active-site thiols, it is possible that two of the three conserved aspartate residues and both conserved histidines are part of a catalytic triad. The occurrence of the strictly conserved Trp425 in the proximity of Asp428 and the conserved dyads Asp480-His481 and Gly507-His508 underscores the likely importance of these residues in catalysis. Analogously, the strict conservation of Pro239, Asn248, Tyr251, and Asp254 in the direct vicinity of the critical Ser260 residues underscores the importance of this stretch of amino acids.

Model for PHA Granule Formation

The resemblance of the active sites of PHA polymerases and lipases, as well as the preferred localization of these enzymes (Fig. 6A), suggests how the process of granule formation may proceed. Both enzymes act on ester bonds at the interface of a hydrophobic vesicle and water. The difference between these enzymes is in the direction of the reaction that they catalyze, either toward ester formation or towards ester hydrolysis. In the aqueous environment of the cytosol, the PHA polymerase is quite a remarkable enzyme since it performs an esterification reaction under typically unfavorable aqueous conditions.

Gerngross and Martin investigated P(3HB) granule formation in vitro and developed a model for P(3HB) granule formation (69). First, soluble P(3HB) polymerase interacts with increasing concentrations of 3-hydroxybutyryl-CoA in the cytoplasm, resulting in priming of the enzyme by an unknown mechanism. During an initial lag phase, HB oligomers are slowly formed and extruded from the enzyme. The HB oligomers then form micelles as the oligomers increase in length and hydrophobicity. Consequently, the micelle-like particles provide a two-phase boundary with the polymerase located at the interface. The enzyme then rapidly proceeds with P(3HB) synthesis, extruding more P(3HB) into the growing granule. Eventually the micelles are thought to coalesce into larger granules that can be visualized by microscopy (69) (Fig. 6B).

In vitro studies of the covalent linkage of the 3HB trimer support this model, since a shift in the conformation of the P(3HB) polymerase from monomer to dimer appeared to coincide with the binding of the trimer. Because the P(3HB) polymerase dimer was more active than the monomer and showed a greatly decreased lag time, it was suggested that the

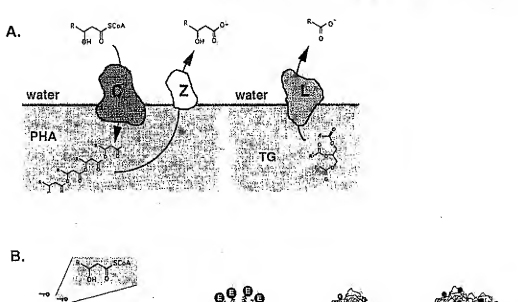

FIG. 6. (A) Similarities between PHA polymerase and lipase. PHA polymerase (C) acts at the surface of a PHA granule, where soluble precursors are polymerized and deposited in the hydrophobic environment of the granule. PHA depolymerase (Z) also acts at this surface and liberates the monomers from the polymer. Both enzymatic reactions are reminiscent of that of lipase (L), which cleaves ester bonds at triglyceride (TG)/water interfaces, yielding free acids and alkanols. (B) Proposed mechanism for the formation of PHA granules. Soluble enzyme converts monomer-CoA to oligomers, which remain enzyme bound (step 1). At a critical oligomer length and enzyme-oligomer concentration, the enzyme-oligomer complexes form micelles with the enzyme located at the interface, separating the PHA from the cytosol (step 2). Because of this compartmentalization, PHA polymerization is facilitated. Because the hydrophobic polymer can now be extruded into a hydrophobic environment instead of the aqueous phase, the reaction proceeds faster. The micelles are expanded and now appear as intracellular, granular structures visible with the phase-contrast microscope (step 3). As the number of granules increase, they may fuse and coalesce, giving rise to large aggregates of PHA (step 4).

lag time in vitro is related to the initial acylation step. It is not yet clear whether this covalent catalysis in the polymerase-catalyzed reaction relates to in vivo priming (296). Physiologically this makes sense, however, since the formation of relatively few high-molecular-weight PHA molecules is expected to be favored over the formation of many low-molecular-weight PHA oligomers. As pointed out above, PHA is considered an osmotically inert macromolecule which depends on having a high molecular weight. Slow PHA polymerase activation in the priming process, combined with a rapid polymerization once activated enzyme forms micelle structures, appears to ensure the formation of high-molecular-weight materials.

The studies by Gerngross and Martin have, furthermore, established that the minimal requirements for P(3HB) synthesis are the (*R*)-3-hydroxybutyryl-CoA substrate and P(3HB) polymerase (69). P(3HB) polymerase is present both in soluble and granule-bound forms, but the soluble P(3HB) polymerase appears less active. Because of the higher activity when granule bound, optimal P(3HB) accumulation occurs when more enzyme is associated with the growing granule. Maintenance of the available surface is thus critical for efficient P(3HB) production. In subsequent studies, Martin and Gerngross observed that the size of in vitro-synthesized granules is related to the amount of protein added to the assay mixture, irrespective of whether this protein is PHB polymerase or an unrelated protein such as bovine serum albumin (161).

PhaP is a natural PHA-binding protein that determines the size of PHA granules. *phaP* was identified in genetic studies as a locus causing a P(3HB) leaky phenotype in *R. eutropha*. The *phaP* gene was cloned from a cosmid library and found to encode a 24-kDa protein that binds to the P(3HB) granule. Immunochemical analysis with anti-PhaP antibodies revealed that the protein is always granule bound and no free PhaP is present in the cytoplasm of the wild-type strain. Genetic studies have furthermore shown that the concentration of PhaP is inversely related to the size of the granule, since overexpression of PhaP resulted in the formation of many small P(3HB) granules while a *phaP* mutant contained only a single P(3HB) granule. The P(3HB) leaky phenotype in *phaP* mutants may therefore be the result of a decreased surface area available for P(3HB) synthesis and causes the observed low polymerase activity (289). This situation indicates an interesting regulatory phenomenon in which maximal activity is obtained by localization of the enzyme to a site which is created and maintained by a structural protein. PhaP is not essential in this regard, but in vivo this protein is likely to be involved in maintenance of the optimal intracellular environment for P(3HB) synthesis and utilization and as such provides guidance during the process of granule formation.

The characteristics of PhaP and related proteins are reminiscent of those of oleosins, proteins that associate exclusively with the oil bodies of oil-producing plants. For that reason, PhaP-like proteins are generally referred to as phasins. It appears that oleosins play a structural role in maintaining the integrity of individual oil bodies by preventing their coalescence (97). Such a role would be especially valuable upon germination of the seeds, when oil degradation is enhanced by a larger surface-to-volume ratio. PhaP and related proteins like GA14 from *Rhodococcus ruber*, GA14 and GA23 from *Methylobacterium rhodesianum*, GA13 from *Acinetobacter*, and the

ORF1 gene product from *Aeromonas caviae* probably have such a function as well and are generally described as phasins (56, 57, 197, 234).

P(3HB)-negative and leaky mutants have been isolated from *R. rubor*, and subsequent immunochemical analysis showed that these phenotypes were both related to aberrant levels of a granule-associated protein, GA14. The absence of GA14 in P(3HB)-negative mutants is likely to be caused by the absolute requirement of the protein to bind P(3HB) granules, as was observed in *R. eutropha*, or by a polar effect on its expression by a *phaC* mutation (Fig. 4) (197). Two carboxy-terminal hydrophobic stretches were shown to be essential for the binding of PhaP to the P(3HB) granules, since PhaP derivatives that lack the two carboxy-terminal hydrophobic domains were unable to do so. This was further supported by the finding that when these carboxy-terminal hydrophobic regions were fused to acetaldehyde dehydrogenase II, the fusion protein localized to the surface of granules in vivo and in vitro rather than to the cytosol (196).

In vitro as well as in vivo studies revealed a role for PHA polymerase in the control of the molecular weight of P(3HB). Variation of the level of PHA polymerase suggested that the concentration of this enzyme is a critical factor in determining the molecular weight of in vitro-synthesized P(3HB). When decreasing amounts of enzyme were supplied to the assay mixture, a polymer was synthesized that had a higher molecular weight (69). New evidence from in vitro studies suggests that P(3HB) formation is a living polymerization in which no chain termination event takes place and that the molecular weight of the resulting polymer is simply dependent on the initial ratio of substrate to enzyme (257). By using an inducer-controlled system to vary PHA polymerase levels in a recombinant *E. coli* strain, the molecular weight of the formed P(3HB) could also be manipulated as a function of the inducer concentration in the culture medium (242).

Other Pathways for scc-PHA Formation

P(3HB) is just one type of the many PHAs that are synthesized by thousands of different microorganisms, all originating from their own ecological niche and with their own evolutionary history. Not all these bacteria use the same biological pathways for PHA biosynthesis, since their metabolic blueprints undoubtedly vary. The three-step P(3HB) pathway involves the reactions catalyzed by thiolase, reductase, and polymerase, as exemplified by *R. eutropha* and *Z. ramigera*. However, some PHA producers use alternative pathways for PHA formation.

In the absence of a thiolase and reductase, *Aeromonas caviae* employs an enoyl-CoA hydratase for the formation of the (*R*)-3-hydroxy monomer from either crotonyl-CoA or hexenoyl-CoA. Other bacteria synthesize P(3HB-3HV) copolyesters from sugars by using a pathway in which 3-HV is derived from the methylmalonyl-CoA pathway. Two additional pathways are found in pseudomonads of rRNA homology group I, which involve either β-oxidation or fatty acid biosynthesis intermediates for mscPHA production. The biosynthetic pathways for the two types of PHAs have therefore diverged at the level of monomer-CoA-supplying routes, while the polymerases evolved to accept either short- or medium-chain monomers. These pathways are discussed in more detail in this section.

PHA synthesis with an enoyl-CoA hydratase. *A. caviae* produces a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HH) when growing on even-numbered fatty acids or olive oil as the sole carbon source. When grown on odd-numbered fatty acids, a PHA is produced that consists primarily of 3HV, but small amounts of 3HB are found as well (45). The crystallinity of a poly(3-hydroxybutyrate-3-hydroxyhexanoate) [P(3HB-3HH)] copolymer decreases from 60 to 18% with an increasing 3HH fraction. This property and its decreased melting temperature make P(3HB-3HH) an interesting polymer for several applications where a material that is more flexible than the P(3HB) homopolymer is desired.

The *pha* locus from *Aeromonas caviae* has been cloned and characterized, shedding light on the metabolic pathway that results in P(3HB-3HH) formation (61, 63). It encodes PHA polymerase (encoded by *phaC*), enoyl-CoA hydratase (encoded by *phaJ*), and a phasin (encoded by ORF1 or *phaP*) and is sufficient for PHA formation in PHB-negative heterologous hosts (61, 63, 234). The identification of PhaJ as an (*R*)-specific enoyl-CoA hydratase suggested that the PHA biosynthetic pathway in *A. caviae* proceeds from enoyl-CoA derivatives of the fatty acid oxidation pathway (Fig. 7). Besides converting crotonyl-CoA to (*R*)-3-hydroxybutyryl-CoA, PhaJ converts pentenoyl-CoA and hexenoyl-CoA to PHA precursors, but it does not convert octenoyl-CoA. It was also shown that some PHA-negative mutants of *A. caviae* are complemented only by *phaJ* whereas others are complemented only by *phaC*. *phaJ* is therefore unique as the first scc-PHA biosynthetic enzyme besides thiolase, reductase, and polymerase (61, 63).

The molecular genetic data on P(3HB-3HH) formation in *A. caviae* provide a new perspective on the work of Moskowitz and Merrick from almost 30 years ago (171). In their work on *Rhodospirillum rubrum*, these authors proposed a pathway for P(3HB) synthesis that included two hydratases, one specific for the *R* enantiomer and the other specific for the *S* enantiomer (171). *R. rubrum* is able to synthesize PHAs from short- and medium-chain fatty acids up to 20% of the cell dry weight. The major monomers are the C_4 and C_6 fatty acids, depending on whether the carbon source has an even or odd number of carbons. Small amounts of C_6 and C_7 monomers were found in PHAs from *R. rubrum* as well (18). Although this pathway has not been paid much attention for many years, it may now see renewed interest in physiological studies on the formation of PHAs composed of both short- and medium-chain 3-hydroxy fatty acids.

Methylobacterium rhodesenium also uses the activities of two hydratases for P(3HB) synthesis (174). In addition to the two hydratases, this bacterium expresses two constitutive acetoacetyl-CoA reductases, one NADH dependent and one NADPH dependent (173). The combination of these four activities may allow for 3-hydroxybutyryl-CoA synthesis under a range of conditions in the absence of a significant transhydrogenase activity. The analysis of key cofactors in cellular metabolism demonstrated that the flux of acetyl-CoA to the tricarboxylic acid (TCA) cycle or to P(3HB) is determined primarily by the CoA levels (175). Interestingly, the growth substrate has a dramatic effect on the timing of the onset of P(3HB) formation in *M. rhodesenium*. During exponential growth on fructose, P(3HB) synthesis is used to prevent the formation of excess reducing equivalents. When methanol is the carbon source, reducing power is not excessive until growth is limited by deficiency of other nutrients and P(3HB) is not formed until the stationary phase (3, 172).

P(3HB-3HV) formation from sugars by the methylmalonyl-CoA pathway. *Rhodococcus ruber* and *Nocardia corallina* accumulate PHAs containing 3HV even in the absence of typical HV precursors such as propionate or valerate in the feed (7, 275). Nuclear magnetic resonance spectroscopy (NMR) studies suggested that the 3HV monomer is derived from acetyl-CoA and propionyl-CoA, where the latter is a product of the

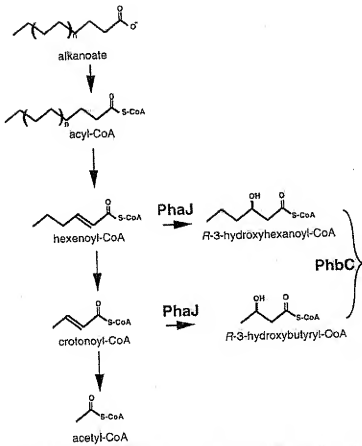

FIG. 7. Biosynthetic pathway for P(3HB-3HH). P(3HB-3HH) monomers are derived from fatty acid degradation by converting enoyl-CoA intermediates directly to (*R*)-3-hydroxyacyl-CoA precursors by an (*R*)-specific enoyl-CoA hydratase (PhaJ).

methylmalonyl-CoA pathway (290). In this pathway, succinyl-CoA is converted to methylmalonyl-CoA, which is decarboxylated to propionyl-CoA (Fig. 8). A mutant strain of *N. corallina* was constructed in which the gene encoding the large subunit of methylmalonyl-CoA mutase was disrupted. The 3HV fraction in the PHAs formed by the resulting mutants was reduced from 70 to 4% compared to that in the wild-type strain. However, the mutants still accumulated P(3HB) on glucose and succinate and a P(3HB-3HV) copolyester on valerate (275). It appears that *N. corallina* derives PHA monomers from both the fatty acid degradation pathway and the traditional P(3HB) biosynthetic pathway, in contrast to *A. caviae*.

Pathways for msc-PHA Formation

msc-PHAs from fatty acids. msc-PHAs were not discovered until 1983, when Witholt and coworkers serendipitously found that *P. oleovorans* grown on 50% octane formed a material that was pliable under conditions where samples are prepared for freeze fracture electron microscopy. Because these materials left mushroom-like structures in the electron micrographs where P(3HB) formed spike structures, further characterization was warranted (41). By using chemically synthesized standards, the inclusions formed from *n*-octane were determined to be made of a copolyester consisting of 89% (*R*)-3-hydroxyoctanoate and 11% (*R*)-3-hydroxyhexanoate (135).

Subsequent studies showed that the composition of the PHAs formed by pseudomonads of the rRNA homology group I were directly related to the structure of the alkane, alkene, or fatty acid carbon source (17, 105, 135). When the carbon source consists of 6 to 12 carbon atoms, the monomers in the PHA are of the same length as the carbon source or have been shortened by 2, 4, or 6 carbon atoms. When the carbon source is a straight-chain C_{12} to C_{18} fatty acid, the composition of the polymer resembles that of the C_{14}- and C_{12}-grown bacteria (105). Use of mixtures of hydrocarbons or fatty acids as the carbon source results in the formation of PHAs in which the composition is a reflection of the ratio of the two carbon sources. For instance, when *P. oleovorans* is supplied with mixtures of octane and 1-octene, the ratio of monomers with an unsaturated bond ranged from 0 to 50% depending on the fraction of 1-octene in the substrate (135). By analogy, substituted 3-hydroxyalkanoates were introduced to different levels by supplying 7-methyloctanoate, 8-bromooctanoate, phenyldecanoate, or cyanophenoxyhexanoate as the cosubstrate (58–60, 85, 124, 126). Incorporation of the last of these substrates results in PHA with monomer constituents that are hyperpolarizable and may confer nonlinear optical properties to the polymer (124).

The composition of these PHAs and their direct relationship with the structure of the growth substrate suggested that the msc-PHA biosynthetic pathway is a direct branch of the fatty acid oxidation pathway (Fig. 9) (135). In this pathway, fatty

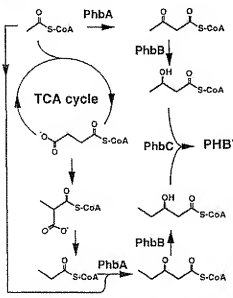

FIG. 8. Biosynthetic pathway for P(3HB-3HV) from carbohydrates. Some microorganisms accumulate P(3HB-3HV) without supplementation of propionate, valerate, or other $C_{>4}$ fatty acids. Propionyl-CoA in these species is formed through the methylmalonyl-CoA pathway, which originates from succinyl-CoA in the TCA cycle. Propionyl-CoA and acetyl-CoA are converted to P(3HB-3HV) by the typical Phb enzymes.

weight of PHA from a depolymerase mutant was not higher than that of PHA from the wild type (106). The latter observation prompted the hypothesis that the molecular weight of PHA is determined by the activity of the PHA polymerase. Based on in vitro analysis of the PHA polymerase from *P. oleovorans*, it has recently been suggested that the substrate is the limiting factor for PHA formation. Overall, these in vivo and in vitro experiments suggest that the substrate/enzyme ratio, and hence the substrate concentration and enzyme levels, determines the molecular weight of the resulting PHA (129, 130).

msc-PHAs from carbohydrates. When fluorescent pseudomonads of rRNA homology group I are grown on sugars, a PHA that consists primarily of C_{10} and C_8 monomers is formed (84, 102, 270). Evidence suggests that these monomers are derived from intermediates of fatty acid biosynthesis and that the composition of the PHAs is probably a reflection of the pool of fatty acid biosynthetic intermediates.

It is well known that temperature affects the fatty acid composition of bacterial membranes. Since this effect is due to enzyme activities in fatty acid biosynthesis, the PHA composition was studied in relation to the growth temperature. When *P. putida* was grown on decanoate, the PHA composition was almost identical irrespective of the growth temperature. In contrast, when glucose was the substrate, the fraction of unsaturated monomers increased from 10 to 20% and the fraction of monomers longer than C_{10} increased from 18 to 28% when the temperature was lowered from 30 to 15°C. Since the

acids are degraded by the removal of C_2 units as acetyl-CoA. The remainder of the pathway oxidizes acyl-CoAs to β-ketoacyl-CoAs via 3-hydroxyacyl-CoA intermediates. The substrate specificity of this msc-PHA polymerase ranges from C_6 to C_{14} (*R*)-3-hydroxy-alkanoyl-CoAs, with a preference for the C_8, C_9, and C_{10} monomers (105). However, because the β-oxidation intermediate is (*S*)-3-hydroxyacyl-CoA, an additional biosynthetic step is required for synthesis of the (*R*)-3-hydroxyacyl-CoA monomer. Whether this PHA precursor is the product of a reaction catalyzed by a hydratase (as in *A. caviae*), by the epimerase activity of the β-oxidation complex, or by a specific 3-ketoacyl-CoA reductase is unknown.

Given the different biosynthetic pathways, it is not surprising that the *pha* loci in the msc-PHA-forming pseudomonads are very different from the *pha* loci in the ssc-PHA-forming bacteria (Fig. 4). Genes involved in msc-PHA formation have been characterized from *P. oleovorans* (107) and *P. aeruginosa* (269). In both species, two closely linked PHA polymerases were identified, and PHA polymerase genes are separated by one open reading frame. The two polymerases are approximately 50% identical in their primary structure and appear equally active in PHA synthesis from fatty acids (106, 107) or glucose (102). The open reading frame between *phaC1* and *phaC2* complements a mutation that prevents the utilization of accumulated PHA. The presence of a lipase box in the primary structure of the product of this gene, *phaZ*, and the homology of the gene product to other hydrolytic enzymes suggest that this gene encodes a PHA depolymerase (107). Downstream of *phaC2* are three genes of unknown function, which may bind to the PHA granules (281).

In vivo experiments with *P. putida* showed that when either of the two PHA polymerase genes (*phaC1* or *phaC2*) was introduced on a multicopy plasmid, the molecular weights of the PHAs decreased. These reductions were not caused by an increase in PHA depolymerase activity, since the molecular

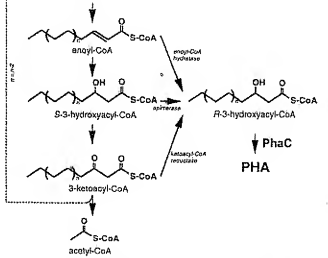

FIG. 9. Biosynthetic pathway for msc-PHA from hydrocarbons. Fluorescent pseudomonads of rRNA homology group I can derive monomers for PHA from fatty acid degradation. Intermediates from the β-oxidation cycle can be converted to (*R*)-3-hydroxyacyl-CoA by a hydratase (H), epimerase (E), or reductase (R) activity, whose nature is currently unknown. Any or all of these three enzymes and PHA polymerase determine the limits to the substrate specificity, which is from C_6 to C_{14} 3-hydroxy fatty acids.

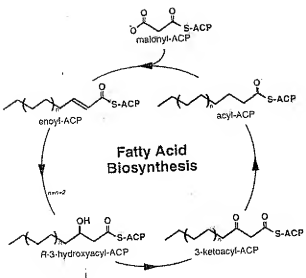

FIG. 10. Biosynthetic pathway for msc-PHA from carbohydrates. Monomers for PHA are derived from the fatty acid biosynthesis pathway as (*R*)-3-hydroxyacyl-ACP intermediates and are converted to (*R*)-3-hydroxyacyl-CoA through an acyl-ACP:CoA transacylase encoded by the *phaG* gene.

ratio of unsaturated to saturated monomers increases at lower temperature for both membrane lipids and PHA, a metabolic relationship between fatty acid biosynthesis and PHA formation from glucose was suggested (102).

Further corroboration of the involvement of fatty acid biosynthesis in PHA formation for glucose and β-oxidation from fatty acids was obtained by inhibition experiments. Nongrowing cultures of *P. putida* are able to synthesize PHA from either glucose or fatty acids when carbon sources are in excess. However when cerulenin (a fatty acid synthesis inhibitor) is added to such cell suspensions, no PHA is formed from glucose whereas PHA is still synthesized from fatty acids. Similarly, acrylic acid, a β-oxidation blocker, prevents the formation of PHA from octanoate but not from glucose (100).

These experiments confirmed that PHA formation from glucose is linked to fatty acid biosynthesis (Fig. 10). Since fatty acid biosynthesis proceeds via (*R*)-3-hydroxyacyl-ACP, a new enzymatic activity was required that converts this intermediate to (*R*)-3-hydroxyacyl-CoA. Recently, Rehm et al. determined that the gene product of *phaG* is responsible for this conversion (214).

Some *Pseudomonas* spp. can incorporate both ssc- and msc-PHA monomers in the same polymer chain. Typically, these PHAs are formed when these strains are grown on unrelated carbon source such as carbohydrates or 1,3-butanediol (2, 116, 139, 255). The PHA polymerases synthesizing these ssc- and msc-PHAs must therefore have a very broad substrate range. This type of mixed PHA is probably exceptional since it has been shown that physical constraints prevent the formation of mixed granules containing both P(3HB) and msc-PHA chains. This was concluded from experiments where a recombinant *P. putida* strain containing both the chromosomal *phaC* and a copy of the *R. eutropha phbC* on a plasmid was shown to accumulate individual granules composed of either P(3HB) or PHA (206, 268). The recent isolation of PHA polymerase genes from *Pseudomonas* sp. strain 61-3, which accumulates P(3HB) and P(3HB)-co-PHA granules from glucose (117), should provide further insights into the simultaneous metabolism of the two types of PHA.

Physiological and Genetic Regulation of PHA Production

The regulation of PHA production is quite complex, since it is exerted at the physiological level, through cofactor inhibition of the enzymes and availability of metabolites, and at the genetic level, through alternative σ-factors, two-component regulatory systems, and autoinducing molecules. Another level of regulation is discussed above and relates to granule size and molecular weight control by levels of PHA polymerase and phasins.

Several leaky mutants of *R. eutropha* that have a phenotype of reduced P(3HB) synthesis have been isolated. Mutations in *phbHI* alter the timing of P(3HB) synthesis, suggesting a regulatory role for the corresponding gene products. Whereas the wild-type strain synthesized P(3HB) to approximately 90%, *phbHI* mutants accumulated P(3HB) to 50% of their dry cell weight, although levels of the P(3HB) biosynthetic enzymes were similar in the wild-type and mutant strains. Upon continued incubation of the mutant strain, the polyester was degraded. This degradation of the polymer was not seen to an appreciable degree in the wild-type strain. The mutant also lacked the ability to transiently secrete 3HB (3 mM maximally), in contrast to the wild-type strain, and secreted pyruvate temporarily up to 8 mM instead (210).

Mapping and nucleotide sequencing of the Tn*5* insertions indicated that the *phbHI* mutants resulted from the inactivation of genes encoding homologs of the *E. coli* phosphoenol-pyruvate phosphotransferase system (PEP-PTS). PhbI has 39% identity to enzyme I of *E. coli* and *Salmonella typhimurium*, while *phbH* encodes a gene product with 35% identity to HPr from *E. coli*, *S. typhimurium*, and *Staphylococcus aureus* (210). The PEP-PTS is involved in the PEP-dependent uptake system of sugars in *E. coli* and *S. typhimurium* (201), but HPr has also been implicated in regulating σ^{54}-directed transcription (216). Pries et al. proposed that this "leaky" phenotype of *phbHI* mutants could actually be caused by aberrant regulation of the P(3HB) degradation pathway and suggested that the activity of the P(3HB)-degrading enzymes was controlled by phosphorylation through metabolic signaling that involves a PEP-PTS (210).

Mutants with mutations in *phaL* compose a second class of leaky mutants of *R. eutropha*. This gene encodes the lipoamide dehydrogenase component of the pyruvate dehydrogenase enzyme complex. The *phaL* mutation resulted in the accumulation of only one-third of the normal amount of P(3HB). Instead of funneling excess carbon into P(3HB) upon nitrogen limitation, this mutant secreted pyruvate up to 33 mM. After the complete consumption of the initial carbon source (fructose), pyruvate was utilized as the carbon source. Apparently the *phaL* mutation results in a decreased flux of carbon into acetyl-CoA and the TCA cycle. As a consequence, the cells do not efficiently metabolize pyruvate upon nitrogen exhaustion and secrete this intermediate. It is of interest that these mutants grow as well as the wild type, as it was expected that a decreased flux through the TCA cycle would affect the growth rate. Although the *phaL* mutation is a Tn*5* insertion within the

gene, the mutant still has residual lipoamide dehydrogenase activity. Indeed, it has been shown that *R. eutropha* has two enzymes that specify this activity. The regulation of these two genes and the role of the second lipoamide dehydrogenase remain to be determined (209).

Azotobacter vinelandii UWD is a mutant strain that synthesizes P(3HB) during growth (184). This strain is impaired in NADH oxidase and uses the NADH-NADP transhydrogenase and P(3HB) synthesis to regenerate NAD during growth (158). The increased NADPH level that results from this mutation causes inhibition of citrate synthase and the TCA cycle. Consequently, acetyl-CoA accumulates and is converted to P(3HB) through the NADPH-dependent pathway. This branch point in acetyl-CoA metabolism to either the citric acid cycle or P(3HB) biosynthesis is also important in *R. eutropha* (89). Park et al. created an increased flux of acetyl-CoA to P(3HB) production by introducing a leaky mutation in the isocitrate dehydrogenase of *R. eutropha* (188). These findings indicate the importance of the redox balance in the cell in the control of PHB formation.

In *Acinetobacter* spp. P(3HB) synthesis is stimulated by low phosphate concentrations. A promoter that might be responsible for this regulation was identified by primer extension analysis and found to contain a sequence that is homologous to the *pho* box identified in *E. coli*. Whereas all three *phb* genes appear to be preceded by a promoter region, the phosphate-inducible promoter is only found upstream of the first gene, *phaB*. This could indicate that for efficient P(3HB) synthesis, the reductase enzyme is limiting and only under conditions of phosphate limitation is the P(3HB) biosynthetic pathway optimally induced (233).

Regulation of PHA synthesis in *Pseudomonas* has been studied to a limited extent. Many pseudomonads are able to synthesize PHAs by two different pathways: through fatty acid biosynthesis when grown on gluconate or through fatty acid degradation when grown on fatty acids. The two PHA polymerases that have been identified in *P. putida* are functional in either of the two biosynthetic pathways (102). In *P. aeruginosa*, the pathway from gluconate is strictly controlled by RpoN, the σ^{54} subunit of RNA polymerase, while the pathway from fatty acids is completely σ^{54} independent (269). In contrast to other mcc-PHA producers, *P. putida* KT2442 synthesizes PHA during exponential growth when grown on fatty acids (106). Recently, the involvement of a two-component system homologous to the sensor kinase/response regulator couple LemA-GacA was found to regulate PHA synthesis in this strain (15). LemA, GacA, and their homologs can sense environmental conditions and relay these signals to control the expression of a diverse set of genes (30, 71, 95, 137, 228, 294). Given the potential role of PHAs in nature as a store of excess carbon and reducing equivalents, it is not unlikely that PHA formation is part of a regulon that is controlled by growth conditions.

The synthesis of P(3HB) in *Vibrio harveyi* is regulated by a 3-hydroxybutyryl-homoserine lactone (258), a signaling molecule that accumulates at high cell densities. A variety of microorganisms regulate the expression of genes at high cell density with such acyl-homoserine lactone derivatives (66). The possible involvement of such signals is consistent with the preferred production of PHAs in stationary phase. Since it was recently shown that GacA homologs and acyl-homoserine lactone derivatives may work through a common signaling pathway (137, 215), the regulatory circuits active on the PHA regulon become more complex. Further studies will clarify whether PHA accumulation is generally regulated by these signals and signal transducers and how environmental information is relayed to the PHA biosynthetic genes.

TABLE 3. Location of *phaQ* with respect to the endogenous PHA polymerase-encoding gene *phaC*

Microorganism	Location of[a]:		% Overlap[b]
	phaC	*phaQ*	
A. caviae	2640–4478	2657–4303	89.6
Acinetobacter	2351–4123	None	
C. vinosum	831–1898	907–1953	92.9
M. extorquens	1099–2236	593–2741	100
N. cornilina	471–2156	551–2587	95.3
P. aeruginosa 1	1266–2945	1472–2935	87.1
P. aeruginosa 2	4259–5941	4687–6096	74.6
P. denitrificans	662–2536	205–1605	50.3
P. oleovorans 1	552–2233	492–1908	80.6
P. oleovorans 2	3217–4950	3093–5063	100
R. eutropha	842–2611	1075–2619	86.8
R. eli	121–2031	48–1400	67.0
R. meliloti	316–2049	<1–1934	93.4
R. sphaeroides	1023–2828	918–2773	97.1
R. ruber	786–2462	119–2419	97.4
Synechocystis	2342–3378	None	
T. violacea	3028–4095	2028–4016	92.6
Z. ramigera	740–2470	733–2373	94.4

[a] The location of the coding regions with respect to the reported *pha* sequences is indicated.

[b] The percent overlap indicates the length of the *phaC* gene that has *phaQ* sequence on the complementary strand as part of the length of *phaC*. It is unknown whether *phaQ* represents coding information for an actual protein or RNA molecule.

A hitherto unnoticed open reading frame (*phaQ*) is located on the opposite strand of all but two of the *phaC* genes (Table 3) (103). It is unknown whether this putative open reading frame is transcribed. Proteins possibly encoded by *phaQ* have no similarity to any other protein in the GenBank database. We can therefore only speculate on a function of this open reading frame, and a protein or RNA originating from this locus could be involved in regulating PHA metabolism.

Maintenance of Redox Balance in Nitrogen-Fixing Bacteria

PHA formation in *Rhizobium* spp. is not commonly studied for reasons of PHA production, but it provides an excellent example of the interplay between cellular metabolism and polyester formation. The symbiosis of *Rhizobium* species with their host plants provides the plant with a system to fix atmospheric nitrogen through the action of the bacterial nitrogenases in the bacteroid. The complex development of *Rhizobium* bacteria from free-living cells to bacteroids inside the plant vacuoles after infection of the plant root system is an important subject of study for the development of more efficient plant crops. Werner et al. have indicated that the activities of the enzymes acting on the amino acid pool of the bacteroid are directly related to the effectiveness of the nodules in nitrogen fixation (288). Bergersen et al. postulated that P(3HB) plays a role in the physiology of bacteroids in the nodule (11). The metabolic activity of the bacteroid is thus critical for the establishment of successful symbiosis.

Transposon mutants of *Rhizobium meliloti* with defects in P(3HB) formation were generated and examined for their effects in symbiosis. The phenotypes of four P(3HB)-negative mutants were similar to that of the wild-type strain with respect to induction of nodule formation on alfalfa (*Medicago sativa*). In addition, the ethylene-reducing activity, a measure of the nitrogenase activity, was also not affected in these *phb* mutants. Such results prompted the conclusion that efficient symbiosis between *R. meliloti* and alfalfa is not affected by alterations in

the P(3HB) metabolic pathways (203). This finding is actually not surprising, given that *R. meliloti* bacteroids typically do not deposit P(3HB) (23).

The inability of *R. meliloti* to form P(3HB) in the bacteroid may be due to low activity of the NADPH-dependent malic enzyme (49). Malate and other four-carbon dicarboxylic acids are provided by the plant and are the preferred carbon sources for the bacteroids (256). In fact, mutants with mutations in either the uptake system for these substrates or the malic enzymes are severely affected in nitrogen fixation. *R. meliloti* has two malic enzymes, one of which is NADH dependent (encoded by *dme*) and the other of which is NADPH dependent (encoded by *tme*). Whereas Dme and Tme are both expressed in the free-living state, Tme expression is repressed specifically in the bacteroid whereas Dme is inhibited by acetyl-CoA. As a consequence, P(3HB) formation is inhibited because too little substrate and too few reducing equivalents are present in the *R. meliloti* bacteroid to pull acetyl-CoA to 3-hydroxybutyryl-CoA (49) (Fig. 11A). Thus, metabolism in *R. meliloti* may have evolved so that P(3HB) is not formed in the bacteroid, since P(3HB) formation does not benefit the symbiosis.

In contrast to *R. meliloti*, *R. etli* does form P(3HB) in both the free-living and bacteroid state. *R. etli* CE3 is auxotrophic for biotin and thiamin, cofactors for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, respectively, and in the absence of these vitamins P(3HB) was accumulated to high levels. As a result of these auxotrophies, the TCA cycle cannot function optimally even in the aerobic free-living state, and the role of the TCA cycle as an overflow mechanism for carbon and reducing equivalents appears to be taken over by P(3HB) formation (53). A P(3HB)-negative mutant of *R. etli* was constructed by insertion of an antibiotic resistance marker in the *phaC* gene. This mutant strain was growth impaired when glucose or pyruvate was the carbon source but not when succinate was the carbon source. On succinate the mutant excreted increased levels of organic acids and had a lower ratio of NAD to NADH compared to the parent strain (23). These data underscore the importance of P(3HB) formation for maintaining the redox balance and supporting a functional TCA cycle.

In contrast to the wild-type strain, nodules of the *R. etli phaC* mutant showed higher and prolonged nitrogenase activity, which fixes atmospheric nitrogen into ammonium ions. As a consequence, plants inoculated with the *phaC* mutants had a higher nitrogen content (23). It was proposed that the increase in reducing equivalents in the absence of P(3HB) formation is used by nitrogenase, similar to a *Rhodobacter sphaeroides* P(3HB)-negative mutant which uses the increased reductive power for hydrogen generation (109). The results obtained with the *R. etli* P(3HB)-negative mutant led to an explanation for the efficiency of alfalfa nodules in nitrogen fixation. These nodules are the result of a symbiosis with phenotypically P(3HB)-negative *R. meliloti*, which leads to an increased availability of reducing power for the nitrogenase enzyme (90). Apparently, nature has evolved the alfalfa-*R. meliloti* symbiosis to improve nitrogen fixation by preventing P(3HB) formation. Why the *R. etli* symbiosis with pea has not selected against P(3HB) formation is a mystery but prompts one to believe that P(3HB) plays another role in this relationship, possibly for survival of *R. etli* in the free-living state (23).

Hahn et al. studied the *nif* region of *Bradyrhizobium japonicum* and found that Tn5 mutants in the nitrogenase-encoding *nifD*, *nifK*, and *nifH* genes resulted in increased P(3HB) accumulation (76). Apparently, the absence of nitrogen fixation in these *B. japonicum* mutants also results in an energy status of the cell that supports increased P(3HB) synthesis. It seems that P(3HB) synthesis serves as an alternative pathway in these mutants for the regeneration of reducing equivalents.

Studies of amino acid uptake mutants in *R. leguminosarum* have also indicated a link between amino acid metabolism and P(3HB) formation (Fig. 11B). A general amino acid permease (Aap), which imports amino acids or exports glutamate, has been identified in this organism. However, when glutamate is secreted, no amino acids are taken up. Mutants with reduced activity of this transporter were isolated based on their resistance to aspartate, and the corresponding mutations were mapped in genes encoding the TCA cycle enzymes succinyl-CoA synthetase (*sucCD*) and 2-oxoglutarate dehydrogenase (*sucAB*). A second class of mutants had mutations in *phaC*, encoding P(3HB) polymerase. The increased secretion of glutamate due to mutations in either the TCA cycle or P(3HB) synthesis prevented aspartate uptake to confer the resistance phenotype. Glutamate therefore appears not to be important as a carbon and energy source; instead, the synthesis and secretion of glutamate is important to balance carbon and reducing equivalents, especially in the absence of a functional TCA cycle or PHB pathway. Because bacteroids are typically anaerobic, the TCA cycle requires cofactor regeneration by other means than oxidation with molecular oxygen. Apparently, both glutamate synthesis and P(3HB) synthesis play this role (283).

In the bacteroid stage, the nitrogen fixation apparatus is competing with P(3HB) formation for reducing equivalents. *Rhizobium* apparently evolved mechanisms to maintain a functional TCA cycle under anaerobic or microaerobic conditions (Fig. 11C). In the bacteroid, the reducing equivalents are used for nitrogen fixation to support symbiosis, but they can be used for P(3HB) formation as well. In the free-living state, nitrogenase is not expressed and P(3HB) plays a role as a sink for excess NAD(P)H when the TCA cycle is not completely active. By regulating the levels of the three different pathways to oxidize NAD(P)H, different *Rhizobium* spp. have evolved a variety of symbiotic conditions.

Conclusions

PHA biosynthesis proceeds through the action of only a few enzymes, which are specifically involved in PHA formation. The genes encoding these enzymes are essential for PHA formation. In addition, a range of other activities affects the amount of PHA that is accumulated, including enzymes that are involved in central metabolism, global metabolic regulation, or control and maintenance of the surface of PHA granules (Fig. 12). Taken together, these molecular genetic data provide a glimpse of the complexity of PHA metabolism. Since PHA formation is dependent on the fluxes in central metabolic pathways and the levels of precursors, a detailed knowledge of the molecular physiology of PHA metabolism is critical for successful implementation of transgenic PHA producers. Unlike the production of heterologous proteins, which relies mostly on sufficient gene expression, recombinant PHA production involves coordinated expression of heterologous enzymes over a prolonged period and with a concomitant redirection of the metabolism of the host. As a consequence of the metabolic changes introduced by expressing the *pha* and *phb* genes, the cell will induce its own responses, which are not necessarily favorable for PHA production. It is therefore critical to understand how bacteria normally regulate PHA formation and how undesired responses from a recombinant host can be prevented. Only then can recombinant processes be

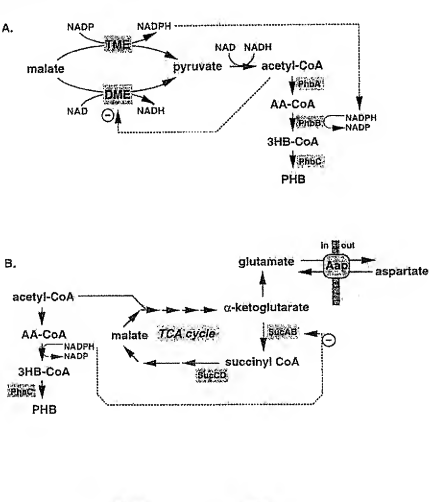

FIG. 11. P(3HB) metabolism and N_2 fixation in *Rhizobium*. (A) In the bacteroid of *R. meliloti* in symbiosis with alfalfa, the Tme malic enzyme is not expressed while Dme is inhibited by excess acetyl-CoA. Consequently, the levels of NAD(P)H are too low to pull acetyl-CoA into the P(3HB) pathway. In the free-living state, however, both Tme and Dme are active and P(3HB) formation is initiated under the desired conditions. (B) A direct link in central metabolism between the TCA cycle, P(3HB) formation, and amino acid metabolism is apparent from studies of the *R. leguminosarum* amino acid permease. Mutants that are less sensitive to high levels of aspartate have an increased secretion of glutamate. This increased production of glutamate is caused by inhibition of the TCA cycle either by a mutation in one of the genes encoding a TCA cycle enzyme or by a mutation in the PHA polymerase gene. In the absence of P(3HB) synthesis, the TCA cycle cannot function optimally, since increased reducing equivalents inhibit α-ketoglutarate dehydrogenase. Both types of mutations cause accumulation of α-ketoglutarate, which is directly converted to glutamate. (C) Recycling of reducing equivalents in *Rhizobium*. The TCA cycle is the most important pathway for supplying precursors of amino acids. To keep the TCA cycle active in the anaerobic bacteroid, P(3HB) biosynthesis and nitrogenase oxidize reducing equivalents. Different *Rhizobium* spp. have evolved different means to regulate the three NAD(P)H-oxidizing pathways in the free-living or bacteroid state.

successfully developed and lead to what are expected to be the most efficient PHA production processes.

PRODUCTION OF PHAs BY NATURAL ORGANISMS

The different examples provided in the previous section illustrate the diversity of the microbial community with respect to different metabolic pathways that are prominent in bacterial species isolated from different sources but that all lead to the formation of PHAs. It is this diversity of pathways that provides the bricks for the construction of an optimal recombinant PHA producer. Those optimal recombinant PHA producers can be evaluated only in the context of the wild-type organisms. Therefore, in this section the state of the art in PHA production by natural organisms is described to provide the back-

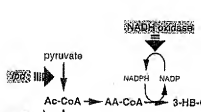

FIG. 12. Ancillary genes encoding enzymes and proteins that affect PHA accumulation. Three enzymes encoded by three genes are essential for P(3HB) formation. Several other gene products, however, affect P(3HB) formation, and mutations in the corresponding genes may decrease P(3HB) levels. Such enzymes and proteins can act on different aspects of P(3HB) formation: monomer supply, cofactor regeneration, granule assembly, or polymer degradation.

ground information needed to assess the merits and prospects of recombinant organisms.

P(3HB) was the only PHA known for almost 50 years until Wallen and Rohwedder (282) identified a number of additional 3-hydroxy fatty acids in active-sludge samples. The major force to commercialize PHAs was Imperial Chemical Industries, Ltd. (ICI), in the 1970s. Several bacterial species were evaluated as potential production organisms. The low cost of methanol and ICI's experience with fermentations of methanol utilizers made methylotrophic bacteria the obvious first choice. However, the amount of polymer produced per cell was insufficient and its molecular weight was too low for the envisaged applications. The second organism of choice was *Azotobacter*, since it was microbiologically well understood and was recognized as a putative production organism. However, the studied strains were unstable and secreted polysaccharides. Obviously, the formation of any by-product is undesirable and should be kept to a minimum since it directly impacts the yield of product. The third organism of choice was *R. eutropha*, which produced high-molecular-weight P(3HB) on fructose. Accumulation of P(3HB) by *R. eutropha* proceeds preferentially under nitrogen- or phosphate-limiting conditions. The resulting production process with this bacterium was in 200,000-liter stirred fermentation vessels (20).

The first copolymer that was produced in fermentation systems also initiated the subsequent surge in interest in PHAs. A patent by Holmes described the controlled synthesis of P(3HB-3HV), in which the 3HV fraction in the polymer could be controlled by the concentration of propionate in the growth medium (92). After the discovery of polyhydroxyoctanoate [P(3HO-3HH)] (Fig. 1) in octane-grown *P. oleovorans* (41), the range of different constituents of PHAs expanded rapidly, and currently close to 100 different PHA monomers have been identified (254).

Comparison of PHA production by different organisms is generally not informative, due to the diversity of PHAs, production organisms, substrates, and growth conditions used by different laboratories. One should also consider that the rationale for the various studies may be different and that the different experimental details render the results not comparable. In sophisticated fermentation systems, higher cell densities can be obtained, which inherently lead to higher productivities per unit volume. In this section, we describe the different procedures that have been used to study the production of PHAs. The results are therefore generally presented in terms of "PHA accumulation as the percentage of the cell dry weight" and "monomer composition as the percentage of the polymer." In general, these studies provide strategies and clues to increase productivities for industrial-scale operations. Production studies with the three most extensively studied organisms are described and are followed by a section on the use of raw but cheap carbon sources for PHA formation by other organisms.

Ralstonia eutropha

R. eutropha was the production organism of choice for ICI in the development of commercial production facilities for P(3HB-3HV) (20). This microorganism grows well in minimal medium at 30°C on a multitude of carbon sources but not on glucose. A glucose-utilizing mutant was therefore selected and used to produce P(3HB-3HV) at a scale of 300 tons per year (21). Chemie Linz GmbH, Linz, Austria, produced P(3HB) from sucrose at up to 1,000 kg per week by using *Alcaligenes latus*. *A. latus* is substantially different from *R. eutropha* and produces P(3HB) during exponential growth, whereas *R. eutropha* does not start PHA formation until stationary phase (79, 96).

The literature on PHA production by *R. eutropha* is somewhat confusing due to the different strains that have been used. The three strains that have been studied most extensively are the original P(3HB) producer H16 (ATCC 17699) and its glucose-utilizing mutant known as 11599 in the NCIMB collection. Other well-studied strains are ATCC 17697[T], *R. eutropha* SH-69, and a natural isolate, *Alcaligenes* sp. strain AK201. *R. eutropha* has been studied intensively for potential copolymer formation to expand the properties range of ssc-PHAs. Two cultivation techniques have generally been used. In batch experiments, both cell growth and PHA formation are examined in the same medium. In nitrogen-free experiments, cells are pregrown in rich medium and then resuspended in a medium lacking a nitrogen source but with the carbon source of choice.

Feeding strategies for PHA copolymer production. The first comonomer that was incorporated into P(3HB) in a defined growth medium was 3HV (92). 3HV can be formed by condensation of propionyl-CoA with acetyl-CoA by β-ketoacyl-CoA thiolase, followed by reduction to 3HV-CoA. By varying the ratio of acetate and propionate in the substrate, *R. eutropha* H16 accumulates P(3HB-3HV) up to 50% of the cell dry weight, with 3HV levels varying between 0 and 45% (46). By using ^{13}C-labeled carbon sources, it was established that the P(3HB-3HV) biosynthetic pathway is through 3-ketoacyl-CoA thiolase, acetoacetyl-CoA reductase, and P(3HB) polymerase. When valerate was supplied as the carbon source to *R. eutropha* NCIMB 11599, the HV fraction in the polymer was 85%. When mixtures of 5-chlorovalerate and valerate were used, terpolyesters were formed containing 3HB, 3HV, and 5HV monomers up to 46% of the cell dry weight and with 52% 5HV monomer (47). *R. eutropha* H16 and *R. eutropha* NCIMB 11599 were directly compared in experiments where butyrate and valerate were used as the carbon source. NCIMB 11599 was able to direct more 3HV monomer to P(3HB-3HV) (90% 3HV) than was H16 (75%). Also, the molecular weight of the polymer produced by NCIMB 11599 was consistently higher. By using ^{13}C-labeled carbon sources, it was established that these fatty acids were converted to P(3HB-3HV) without undergoing complete degradation to acetyl-CoA and propionyl-CoA. This means that either the (*S*)-3-hydroxyacyl-CoA or 3-ketoacyl-CoA is directly converted into monomer. Interestingly, this pathway operates in the presence of a nitrogen source, in contrast to the pathway from fructose (48). It is possible that inhibition of thiolase during active metabolism of carbohydrates prevents P(3HB) formation during growth

whereas a pathway that involves only reductase and polymerase is insensitive to this inhibition.

R. eutropha H16 accumulates copolyesters of 3HB and 4-hydroxybutyrate (4HB) from mixtures of butyrate and 4HB (132) or mixtures with 4-chlorobutyrate, 1,4-butanediol, or γ-butyrolactone (131). With such mixtures of carbon sources, PHA levels reach 40% of the cell dry weight with 4HB levels up to 37%. As a result of the increased 4HB fraction, a lower melting temperature, a decreased crystallinity (132), and an enhanced rate of PHA degradation are obtained (131). Mixtures of butyrate, valerate, and 4HB led to the accumulation of a P(3HB-4HB-3HV) terpolymer with up to 45% 4HB and 23% 3HV (132). Even higher incorporation levels were achieved with mutants of *R. eutropha* H16 that cannot use valerate or 4HB as the carbon source. When such mutants are tested for copolymer formation, up to 96% 3 HV and 84% 4HB are incorporated (127). Although the total amount of accumulated PHA may be smaller in such mutants, they have great promise for further use in controlled fermentation systems where another carbon source is available to support growth.

Alcaligenes sp. strain AK201 has been studied for P(3HB-3HV) formation on C_2 to C_{12} fatty acids. P(3HB) was formed up to 55% of the cell dry weight on C_{even} fatty acids, whereas P(3HB-3HV) was formed on C_{odd} substrates. As expected, the 3HV content of the polymer was higher on the shorter fatty acids. On plant oils and animal fats, P(3HB) levels were also around 50% of the cell dry weight. Interestingly, the molecular weight of the PHA formed was carbon source dependent and was maximal for C_{7-9} and C_{13-16} fatty acids (5). On dicarboxylic acids in the C_4 to C_9 range, P(3HB) homopolymer was accumulated to 50 to 60% of the cell dry weight (4). Further optimization of P(3HB) production on fatty substrates led to polymer levels over 60% of the cell dry weight in a palm oil fed fermentation. On the other hand, oleate, which is the main constituent of palm oil, supported P(3HB) formation to only 42% of the cell dry weight, and this polyester had a lower molecular weight (157). Apparently, palm oil and the free fatty acid that constitutes the oil have a sufficiently different effect on the cells, leading to variations in PHA productivities. Even though these two carbon sources are degraded by the same metabolic pathway, their nature (ionic/soluble or neutral/insoluble) affects PHA formation.

Copolymer production from central metabolites. At high concentrations, short-chain fatty acids such as propionate and valerate are toxic for *R. eutropha*. Alternative means of introducing 3HV monomers have therefore been explored. Propionyl-CoA is an intermediate in the degradation pathway of threonine, valine, and isoleucine, and strains with mutations in these pathways were tested for P(3HB-3HV) production. *R. eutropha* R3 is a prototrophic revertant of an isoleucine auxotroph of *R. eutropha* H16 and accumulates P(3HB-3HV) with up to 7% 3HV on fructose, gluconate, succinate, acetate, and lactate. To compensate for a threonine dehydratase mutation, *R. eutropha* R3 overproduces acetolactate synthase and secretes valine and some leucine and isoleucine. Under nitrogen-deficient conditions, however, the precursors of these amino acids, 2-keto-3-isovalerate and 2-keto-3-methylvalerate, are overproduced and subsequently degraded through the propionyl-CoA intermediate (251) (Fig. 13).

Addition to threonine, isoleucine, and valine to cultures of *R. eutropha* SH-69 resulted in the incorporation of 53, 41, and 15% 3HV, respectively. Whereas threonine is toxic at high concentrations and consequently reduces biomass and PHA production, isoleucine and valine are not toxic up to concentrations of 50 mM. When the concentration of amino acid supplements exceeds 10 mM, the fraction of 3HV in the polymer is directly related to the concentration of the amino acid. In contrast, *R. eutropha* NCIMB 11599 does not incorporate 3HV from threonine and incorporates only up to 2% from isoleucine or valine (302). When *R. eutropha* H16 was resuspended in Na^+- or O_2-limiting medium with threonine as the sole carbon source, 6% PHA with 5% 3HV was accumulated (176).

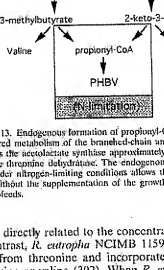

FIG. 13. Endogenous formation of propionyl-CoA in *R. eutropha* R3, which has altered metabolism of the branched-chain amino acids. This mutant overproduces the acetolactate synthase approximately 15-fold to compensate for a defective threonine dehydratase. The endogenous accumulation of propionyl-CoA under nitrogen-limiting conditions allows this strain to produce P(3HB-3HV) without the supplementation of the growth medium with propionate or other cofeeds.

These types of experiments prove that alternative, cell-derived substrates can be used for P(3HB-3HV) synthesis and that supplementation of carbon sources for alternative PHA monomers can be circumvented. Metabolic engineering of new PHA monomer biosynthetic pathways such as from the threonine pathway can thus lead to new P(3HB-3HV)-producing strains. The pathways involved in the biosynthesis of threonine, isoleucine, and valine are well characterized in *E. coli* and other amino acid producers, and engineered *E. coli* strains that produce 79 g of threonine per liter are commercially exploited (37). The combination of developments in metabolic engineering of amino acid and PHA pathways provides a tremendous benefit for the successful generation of economic P(3HB-3HV) producers. It is therefore to be expected that other biotechnological processes will aid in the production of some specific PHAs as well.

Fed-batch and continuous culture. The preceding paragraphs show that the composition of scc-PHAs is determined by multiple factors. The substrate for growth and PHA formation is an obvious parameter. More important is that central metabolism, especially amino acid metabolism, plays an important role. Recognition of such phenomena allows the metabolic engineer to design PHA-producing strains able to accumulate materials with a number of different compositions. The next paragraphs describe in more detail how *R. eutropha* is grown to obtain PHAs in large quantities from different carbon sources.

R. eutropha NCIMB 11599 has been studied intensively in high-cell-density fermentation studies. To reach cell densities of 100 g/liter, a fed-batch mode is the preferred way of operation. In fed-batch fermentations, the addition of nutrients is triggered by specific changes in the growth medium as a result of depletion of one of the required medium components. By using a pH-regulated system for glucose supplementation, P(3HB) was produced to 10 g/liter or 17% of the biomass at a productivity of 0.25 g/liter/h. Because the pH increase in response to carbon limitation is slow for this strain, improve-

ments were sought by using the dissolved-oxygen value as the trigger for further glucose addition (DO-stat). When nitrogen was made limiting at a biomass of 70 g/liter and using a DO-stat, P(3HB) was produced to 121 g/liter, corresponding to 75% of the biomass, with a productivity of 2.42 g/liter/h. The yield of P(3HB) was 0.3 g/g of glucose (121). Since pH control under nitrogen-limiting conditions is achieved by the addition of NaOH, problems occur at high densities in large volumes because of the toxicity of highly concentrated hydroxide (230). In addition, it is very important to maintain phosphate and magnesium ion levels above 0.35 g/liter and 10 mg/liter, respectively (8). Ryu et al. therefore studied P(3HB) formation under phosphate-limiting conditions where the pH is controlled by ammonium hydroxide. Under these conditions, P(3HB) levels of 232 g/liter (80% of the cell dry weight) were obtained with a productivity of 3.14 g/liter/h (230). *R. eutropha* NCIMB 11599 was subsequently grown on tapioca hydrolysate (90% glucose) as a potential cheap carbon source, but unfortunately the presence of toxic compounds, possibly cyanate, in the substrate limited productivity to 1 g/liter/h for a 60-h fermentation (120).

Continuous-culture studies have shown that the theoretical maximal yield of P(3HB) on glucose (0.48 g/g) can be approached to within 5% at a growth rate of 0.05 h^{-1} (88). Such studies also indicated the importance of the growth rate on 3HV incorporation when a fructose-valerate mixture was used as the substrate (128). At dilution rates varying from 0.06 to 0.32, the 3HV content increased from 11 to 79%. Because the toxicity of propionate is pH dependent, P(3HB-3HV) copolymers with different 3HV contents can be produced by varying the pH of the culture as well (27). As described above, *R. eutropha* SH-69 accumulates P(3HB-3HV) from glucose as the only carbon source. For this strain, the 3HV fraction in the copolymer is strongly dependent on the glucose concentration in the medium. Maximal accumulation of P(3HB-3HV) occurs with 2 to 3% glucose and a dissolved oxygen concentration of at least 20%. Unfortunately, a 20% 3HV content is not obtained until 6% glucose is supplied (226). *R. eutropha* DSM 545 produces P(3HB-3HV) from glucose and propionate in fed-batch fermentations under conditions of nitrogen limitation and low dissolved oxygen concentrations. The yield of 3HB on glucose is independent of the dissolved-oxygen concentration, but the HV content is lower at high than at low dissolved-oxygen concentrations (21 and 29%, respectively) (151). The optimal conditions for 3HV incorporation appear to be determined by multiple parameters. As a consequence, the P(3HB-3HV) composition will be influenced to a large extent by the design and setup of the complete process.

Methylobacterium

Methanol is a relatively cheap carbon source and therefore is potentially useful as a substrate for PHA formation (204). Suzuki et al. demonstrated the feasibility of this concept in a series of experiments on P(3HB) formation by *Protomonas extorquens* sp. strain K (259–262). In a fully automated fed-batch culture, 136 g of P(3HB) per liter was formed in 175 h with a yield of 0.18 g of PHB per g of substrate. This polymer had a molecular mass of 300,000 Da. Improvement of the medium composition increased producing to 149 g/liter in 170 h (260, 261). The effect of physiological parameters such as temperature, pH, and methanol concentration were subsequently studied under the optimized conditions (259). When the growth temperature and pH were drastically different from the optimal conditions (30°C at pH 7.0), the molecular weight of the produced P(3HB) was significantly higher. However, such conditions also resulted in a dramatically reduced yield of P(3HB). The methanol concentration, on the other hand, proved to be a useful parameter for molecular weight control. At methanol concentrations of 0.05 to 2 g/liter, P(3HB) was deposited to 50 and 60% of the cell dry weight with molecular masses ranging from 70,000 to 600,000 Da. At higher methanol concentrations, the yield dropped to 30% and the molecular mass dropped to 30,000 Da (259). By using a slow methanol feed to prevent oxygen limitation in a fed-batch fermentation, P(3HB) was accumulated to 43% of the cell dry weight corresponding to 0.56 g/liter/h with a yield on methanol of 0.20 (14). As a result of the slow feed, a molecular mass of over 1,000,000 Da could be obtained.

By using a natural isolate of *Methylobacterium extorquens*, P(3HB-3HV) copolymers were produced from methanol-valerate mixtures. The optimal fermentation conditions consisted of a methanol concentration of 1.7 g/liter, and the addition of a complex nitrogen source. Under these conditions, P(3HB) was accumulated to 30% of the cell dry weight with a molecular mass of 250,000 Da (13). Still other isolates such as *Methylobacterium* sp. strain KCTC0048 have been studied for copolymer synthesis. This organism accumulates P(3HB-3HV), P(3HB-4HB), and poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) {P[3HB-3HP]} to 30% of the cell dry weight with fractions of 3HV up to 0.7, 4HB up to 0.13, and 3HP up to 0.11 (115).

Whereas *M. extorquens* incorporates the methanol-derived formic acid into the serine pathway, another PHA producer, *P. denitrificans*, reduces formation to CO_2, which is subsequently fixed by the ribulose bisphosphate pathway. Interestingly, these different pathways have clear effects on P(3HB-3HV) formation by these organisms (272). *M. extorquens* synthesizes 50% more PHA than *P. denitrificans*, while the latter incorporates twice as much 3HV on methanol-pentanol mixtures. The 3HV fraction in the PHA produced by *P. denitrificans* reaches 0.84 and is based on a relatively small amount of 3HB precursor. Under controlled growth conditions with pentanol as the only growth substrate, *P. denitrificans* accumulates PHV as a homopolymer up to 55% of its cell dry weight (300).

Pseudomonas

The P11A biosynthetic machinery of *P. putida* is most active toward monomers in the C_6 to C_{10} range. Because long-side-chain fatty acids such as oleate ($C_{18:1}$) need to be converted in multiple rounds of the β-oxidation pathway before the resulting C_8 and C_{10} monomers can be incorporated, these substrates are less efficiently converted to PHA than is octanoate. Oleic acid, for instance, has to yield 4 acetyl-CoA molecules before a C_{10} monomer can be incorporated. This conversion yields 20 ATP equivalents in the reduction steps, which is unlikely to occur at a time when excess energy cannot be dissipated. In contrast, decanoic acid and octanoic acid yield 2 ATP equivalents before being incorporated into mcl-PHA. As a consequence, the polymer yields per cell are often higher when medium-chain fatty acids are used. Unfortunately, medium-chain fatty acids are generally more expensive, and therefore a compromise between substrate price and conversion yield is being sought.

mcl-PHA formation by *Pseudomonas* from fatty acids. Inexpensive substrates have been tested for PHA production by *Pseudomonas* species. Tallow is an inexpensive fat that suffers a production overcapacity. Since it is a mixture of triglycerides with oleic, stearic, and palmitic acids as major fatty acid components, tallow represents an interesting substrate for PHA production. Although some of the better characterized *Pseudo-*

monas strains convert hydrolyzed tallow to PHAs at levels between 15 and 20% of their cell dry weight, these organisms do not secrete a lipase enzyme to facilitate tallow hydrolysis. *P. resinovorans*, however, provides both lipase activity and PHA biosynthetic capacity up to 15% of the cell dry weight (31). Whereas tallow is a widely available feedstock in the United States, other countries such as Malaysia have other carbon sources available for PHA production. Studies by Tan et al. (66) show that *P. putida* can convert saponified palm kernel oil to PHA. The major fatty acid constituents of palm oil are lauric and myristic acid (>55%). Whereas PHA from either lauric or myristic acid is semicrystalline, PHA from either oleate or saponified palm kernel oil is amorphous (266). Besides their lowest cost, long side-chain fatty acids offer an additional advantage, since they often contain functional groups that make the resulting PHA amenable to modification after isolation (52). The presence of double bonds in some fatty acids results in unsaturated monomers that provide sites for chemical modification of the PHA. When hydrolyzed linseed oil was used, PHA was accumulated up to 20% of the cell dry weight, with 51% of the monomers being polyunsaturated. The primary fatty acids in linseed oil are linolenic acid, oleic acid, and linoleic acid, and these substrates result in monomers with up to three unsaturated bonds. Interestingly, the initial PHA preparation was amorphous, but exposure to air for 3 days resulted in solidification of the material due to cross-linking of the polyunsaturated monomers (22).

Fed-batch and continuous culture. The yield of PHA on glucose is relatively poor, and production of PHA by fermentation has therefore focused on using fatty acids and hydrocarbons. Initial fermentation studies of *P. oleovorans* on octane showed that cell growth is limited by the oxygen supply. When the growth rate was lowered by decreasing the growth temperature, a higher cell density was obtained (205). With the data from such batch experiments, fed-batch fermentations resulted in a final biomass of 37 g/liter, 33% of which is PHA with a productivity of 0.25 g of PHA/liter/h. Because octane is a flammable substrate, other production studies mostly involved the use of octanoic acid as the carbon source. By using pure oxygen, *P. oleovorans* was grown on octanoic acid to a cell density of 42 g/liter, accumulating 37% PHA with a productivity of 0.35 g/liter/h (145). In an experiment where cells were pregrown on a rich medium followed by resuspension in nitrogen-free minimal medium with octanoate, Hori et al. examined the effect of several physiological parameters on PHA production by *P. putida* (93). The rate of PHA formation is highest at 30°C with an octanoate concentration of 3.5 mM and a pH of 7.8. The molecular mass of the PHA is unchanged over the length of a fermentation process, but both lower temperature (15°C) and a lower octanoate concentration (1.5 mM) give a twofold-higher molecular mass (2.4×10^5 Da). Under these conditions in a two-stage fed-batch fermentation, the yield on octanoate was 0.3 and PHA was accumulated up to 50% of the cell dry weight (93).

Kim et al. studied the effects of the usage of separate carbon sources for growth and PHA production (123). With the use of octanoic acid throughout the fermentation, 25 g of PHA/liter was obtained at a yield of 0.28 g of PHA/g of octanoate. When glucose was used to obtain a biomass of 30 g/liter followed by the supplementation of octanoate for PHA production, the PHA concentration decreased to 18.6 g/liter although the yield was improved to 0.4. The simultaneous supply of both glucose and octanoic acid resulted in 35.9 g of PHA/liter (65% of the cell dry weight) with a high yield (0.4 g/g) and a productivity of 0.92 g/liter/h (123). From these experiments, it appears that mixtures of cheap growth substrates and more expensive substrates for product formation provide a valuable means of lowering PHA production costs. Because oleate is a cheaper substrate than octanoate, its use in a fed-batch production process was studied. Oleate supply was regulated by a DO-stat, and biomass was formed to 92 g/liter, of which 45% was PHA, in only 26 h. This resulted in the production of 1.6 g PHA/liter/h (100).

These studies show the tremendous impact of the growth conditions on PHA formation. Besides these fed-batch studies, optimization of PHA formation was also studied in continuous culture. Although continuous cultures are not industrially feasible and rarely reach the densities of fed-batch cultures, they often provide useful information for the scale-up of production processes.

At low biomass concentrations and a generation time of 0.1 generation/h, *P. oleovorans* produced PHA at a rate of 0.20 g/liter/h on either octane (207) or octanoate (213). Improvements in the medium composition led to a higher productivity (0.56 g/liter/h), primarily because of a higher biomass concentration (205). Similar studies by Huijberts and Eggink describe PHA production on oleate. The highest volumetric productivity obtained was 0.69 g/liter/h at a generation time of 0.1 h^{-1} (101). Although these productivities are lower than those obtained in fed-batch cultures, the data show the importance of the growth medium and give an indication of the optimal generation time during the later stages of growth.

PHA formation by *Pseudomonas* from carbohydrates. Initially it was surprising when *P. putida* strains were found to be able to accumulate PHAs from glucose and other sugars. The first mcl-PHA producer, *P. oleovorans*, was unable to do so, and it was expected that the mcl-PHA pathway would be exclusively fatty acid based. However, several studies showed that *P. putida* and *P. aeruginosa* strains are able to convert acetyl-CoA to medium-chain-length monomers for PHA synthesis. In fact, it now turns out that rather than being the rule, *P. oleovorans* is an exception among the pseudomonads in being unable to synthesize PHAs from sugars. PHAs that are formed from gluconate or related sugars have a different composition from the PHAs from fatty acids. Whereas the latter PHAs have 3-hydroxyoctanoate as the main constituent, sugar-grown cells accumulate PHAs in which 3-hydroxydecanoate is the main monomer and small amounts of unsaturated monomer are present (84, 102, 270).

PHA Production by Other Microorganisms

PHA producers have been isolated from several waste stream treatment sites, since these facilities often provide a mixture of substrates that select for a variety of organisms. In addition, waste streams often contain high concentrations of organic molecules such as fatty acids, which are inexpensive substrates for PHA formation. Several investigators have studied PHA production by natural isolates from genera such as *Sphaerotilus*, *Agrobacterium*, *Rhodobacter*, *Actinobacillus*, and *Azotobacter* to convert organic waste into PHA.

Sphaerotilus natans is a typical inhabitant of activated sludge, where it is associated with the common problem of poor settling of the sludge. Wild-type isolates of this bacterium produce P(3HB) up to 30% of the cell dry weight, but mutants unable to form its encapsulating hydrophilic sheath overproduce P(3HB) up to 50% (265). The P(3HB)-overproducing mutant was found to be tolerant to 6 g of propionate per liter, which is at least sixfold higher than for *R. eutropha*. Consequently, *S. natans* is considered an excellent candidate for P(3HB-3HV) synthesis from glucose and propionate mixtures. The high concentration of propionate that can be supplied to

the culture facilitates the fermentation process. The 3HV content and the final amount of PHA accumulated are pH dependent in this bacterium. The 3HV fraction varies from 15 to 43% between pH values of 7.3 and 5.9, establishing an additional means of controlling PHA composition besides substrate concentration. Under optimal conditions, PHA was accumulated to 67% of the cell dry weight (264).

Agrobacterium sp. strains SH-1 and GW-014 were isolated from activated sludge as organisms that accumulate P(3HB-3HV) from glucose. Depending on the carbon source, accumulation levels of 30 to 80% PHA with 3 to 11% 3HV were obtained. PHA yields of over 65% with 2 to 6% 3HV were obtained with hexoses such as glucose, fructose, mannitol, and sucrose. On the other hand, PHAs with 8 to 11% 3HV were accumulated when the pentose sugars arabinose and xylose were carbon sources, but only to 35% of the cell dry weight. The propionyl-CoA for 3HV formation is derived from succinate through the methylmalonyl-CoA pathway. It was shown that the specific production rate of the 3HV monomer was dependent on the concentration of Co^{2+} ions, which form part of the vitamin B_{12}-dependent methylmalonyl-CoA mutase. Fed-batch cultivation on glucose-propionate resulted in PHA formation up to 75% of the dry cell weight with 50% 3HV monomer (140).

Rhodobacter sphaeroides has been studied for the formation of PHA from anaerobically treated palm oil mill effluent (POME). In Malaysia, POME is treated primarily such that the organic acids are converted into methane, which is released into the atmosphere. By combining processes in which POME is converted anaerobically to organic acids, followed by PHA production from these acids by a photosynthetic bacterium, carbon sources in the effluent can be converted to PHA (80).

Actinobacillus sp. strain EL-9 has been isolated from soil and accumulates PHA during the logarithmic growth phase. This strain was studied for the conversion of the reduced sugar components in alcoholic distillery wastewater to PHA. This waste stream is rich in sugar and nitrogeneous compounds, which have a high biological oxygen demand (BOD). Lowering of the BOD of this effluent by using it for PHA formation seems an environmentally sound solution for the treatment of this waste stream while simultaneously producing a useful material. Because *Actinobacillus* does not require nutrient-limiting conditions, P(3HB) can be formed continuously on the wastewater stream. Comparative studies of different carbon sources showed that enzyme-hydrolyzed alcoholic distillery wastewater gave the highest conversion of its components to biomass (4.8 g/liter), 47% of which is P(3HB) (246).

Azotobacter vinelandii was recognized early on for its ability to produce P(3HB) (20). *A. vinelandii* UWD was described as a strain that produces P(3HB) during growth, possibly as a result of a defective respiratory NADH oxidase (184). This strain was studied for P(3HB) formation on complex carbon sources such as corn syrup, cane molasses, beet molasses or malt extract (183), fatty acids (185) or swine waste liquor (24). With these different carbohydrates as growth substrates, similar levels and yields of P(3HB) were obtained. Perhaps the unrefined substrates have additional beneficial effects on the fermentation process, since they could promote growth (183). Swine waste liquor consists primarily of acetate, propionate, and butyrate and requires a high BOD. *A. vinelandii* UWD produces P(3HB-3HV) from twofold-diluted swine waste liquor, but the productivity can be remarkably increased by supplementation of additional carbohydrate sources (24).

Initially, the formation of polysaccharides by *A. vinelandii* was considered such a disadvantage that continuing exploration of this organism for commercial P(3HB) production was halted (20). In fact, it has been shown that the synthesis of alginate and P(3HB) are interrelated since they play a role in the response of the cell to growth conditions (19). The amounts of alginate and P(3HB) formed by *A. vinelandii* are dependent on the oxygenation, since a small amount of aeration promotes P(3HB) synthesis over alginate synthesis. The advent of genetic engineering since the initial efforts by ICI has provided mutants of *A. vinelandii* with diminished alginate formation. P(3HB) accumulation levels in these strains were increased from 46 to 75% of the cell dry weight, with a threefold higher yield on sucrose (162). This finding illustrates how modern molecular biological techniques can potentially have a direct impact on industrial P(3HB) production, as is discussed further in subsequent sections.

Conclusions

To discuss in great detail the vast number of organisms capable of producing PHAs would be beyond the scope of this review. The many PHA producers and the structures of the approximately 100 different monomers have been summarized previously (142, 254). It should be clear, however, that the study of the biosynthetic pathways of these diverse organisms provides insight into the processes necessary to engineer accumulation of a variety of PHA polymers in transgenic organisms. In addition, the study of mutants defective in PHA production will aid in identifying the genes required to efficiently express *pha* genes in heterologous organisms, such as *E. coli* and plants. Currently, molecular data on the PHA biosynthetic pathways from over 25 different bacterial species is available. These microorganisms, with their own unique metabolic versatility, provide the foundation from which engineered strains for the production of PHAs can be designed. Not only is this approach useful for recombinant bacterial strains, but also it will be indispensable for further development of a plant crop-based PHA production system.

PHA PRODUCTION BY RECOMBINANT BACTERIA

For the successful implementation of commercial PHA production systems, it is a prerequisite to optimize all facets of the fermentation conditions. The price of the PHA product will ultimately depend on parameters such as substrate cost, PHA yield on the substrate, and the efficiency of product formulation in the downstream processing. This means that high PHA levels as a percentage of the cell dry weight are desirable, as well as a high productivity in terms of gram of product per unit volume and time (38, 40).

Whereas natural PHA producers have become accustomed to accumulating PHA during evolution, they often have a long generation time and relatively low optimal growth temperature, are often hard to lyse, and contain pathways for PHA degradation. Bacteria such as *E. coli* do not have the capacity to synthesize or degrade PHAs; however, *E. coli* grows fast and at a higher temperature and is easy to lyse. The faster growth enables a shorter cycle time for the production process, while the higher growth temperature provides a cost saving associated with cooling of the fermentation vessel. The easier lysis of the cells provides cost savings during the purification of the PHA granules. This section gives an overview of the efforts to construct better PHA producers by applying the insights of genetic and metabolic engineering. The effects of altered expression levels of *pha* genes on PHA formation have been studied in natural PHA producers and are described first.

Recombinant Natural PHA Producers

Several studies report on the effects of additional copies of *phb* or *phu* genes on the formation of polymer by the wild-type organism. Although elevated levels of PHA were occasionally found, no dramatic effects of high-copy-number *phu* genes on PHA metabolism were observed. Such results are consistent with the multilayered regulation of PHA biosynthesis.

When the *phu* genes from *P. oleovorans* were introduced into itself or into *P. putida*, no increased PHA synthesis was observed. The only effect of additional copies of the PHA polymerase-expressing genes was a slight change in the composition of the polymer (107) and a decrease in its molecular weight (106). P(3HB) production in a *Rhizobium meliloti* P(3HB) mutant is also restored to only wild-type levels by a plasmid-encoded *R. meliloti phbC* gene (271), whereas an additional *P. denitrificans phaC* gene on a plasmid doubles the wild-type PHV levels in a pentanol-grown parent strain (273).

In recombinant *R. eutropha* strains that overexpress the *phbCAB* genes from a plasmid, the P(3HB) levels are increased from 33 to 40% of the cell dry weight (189). This small increase appears low in comparison to the 1.5- to 3-fold increase in the levels of the individual enzymes and suggests a major influence of the central metabolic pathways on P(3HB) formation. Subsequent studies with these strains in fed-batch cultures indicated that the use of recombinant *R. eutropha* strains could reduce the fermentation time by 20% while maintaining the same productivity (187). This reduction in fermentation time is significant for commercial production, since the overall productivity for a P(3HB) plant would be 20% higher.

The *phbCAB* operon from *R. eutropha* was also expressed in several *Pseudomonas* strains that normally do not accumulate P(3HB). The plasmid used in these studies expressed the genes successfully, since P(3HB) was deposited by *P. aeruginosa*, *P. putida*, *P. oleovorans*, *P. syringae*, and *P. fluorescens*. In contrast, the non-PHA producer *P. stutzeri* was unable to synthesize P(3HB) with the *R. eutropha* genes (253). Recently, PHB accumulation up to 25% of the cell dry weight was achieved in a recombinant *Synechococcus* sp. containing the *phb* genes from *R. eutropha*. PHB production was significantly enhanced under nitrogen-limiting conditions and with acetate as the carbon source, yielding a polymer with a molecular mass of 465,000 Da (263).

Recombinant *E. coli* as PHA Producer

The availability of a large number of PHA biosynthetic genes facilitates the construction of recombinant organisms for the production of P(3HB). Although *R. eutropha* is an excellent producer of P(3HB), this bacterium has certain limitations that prevent it from being useful for the commercial production of P(3HB). For instance, it grows slowly and is difficult to lyse. In addition, it is not well characterized genetically, which impedes its further manipulation for improved industrial performance. P(3HB) production with recombinant systems may be able to overcome these obstructions. Recombinant *E. coli* could potentially be used to address these problems, since it is genetically well characterized. P(3HB) production in *E. coli* must be engineered, because this organism does not naturally synthesize P(3HB) granules. Since the first *phb* genes were expressed in *E. coli* (192, 236, 245), a variety of other polymers, such as P(3HB-3HV), P(3HB-4HB), P4HB, and P(3HO-3HH), have been synthesized by *E. coli* following genetic and metabolic engineering.

P(3HB). The first indication that P(3HB) could be synthesized in heterologous hosts was obtained when the *phb* genes from *R. eutropha* were cloned in *E. coli* and directed the formation of P(3HB) granules (192, 236, 245). Subsequent reports on cloning of *phb* genes from other prokaryotes often included similar heterologous expression studies. Even though recombinant *E. coli* is able to synthesize P(3HB) granules, these strains lack the ability to accumulate levels equivalent to the natural producers in defined media. The first P(3HB) production experiments in fed-batch cultures therefore were in Luria-Bertani (LB) broth, and P(3HB) levels of 90 g/liter were obtained in 42 h with a pH-stat controlled system (122).

In a comprehensive comparison of recombinant *E. coli* P(3HB)-producing strains, Lee et al. studied 10 different strains equipped with a *parB*-stabilized *phbCAB* plasmid (147). Among wild-type strains, *E. coli* B accumulated P(3HB) to 76% of the cell dry weight on 2% glucose–LB medium, while *E. coli* W, K-12, and EC3132 formed P(3HB) to only 15 to 33% of the cell dry weight. Typical cloning strains such as XL1-Blue, JM109, and HB101, on the other hand, accumulated P(3HB) to levels varying from 75 to 85% of the cell dry weight. By using stabilized plasmids derived from either medium- or high-copy-number plasmids, it was shown that only high-copy-number vectors support substantial P(3HB) accumulation in *E. coli* XL1-Blue (146). In a fed-batch fermentation on 2% glucose–LB medium, this strain produced 81% P(3HB) at a productivity of 2.1 g/liter/h (149). The P(3HB) productivity was reduced to 0.46 g/liter/h in minimal medium but could be recovered by the addition of complex nitrogen sources such as yeast extract, tryptone, Casamino Acids, and collagen hydrolysate (144). By supplementing different amino acids separately, it was apparent that P(3HB) formation in recombinant XL1-Blue is limited by available NADPH. Addition of either amino acids or oleate, both of which require substantial reducing equivalents for their synthesis, generally increased cellular P(3HB) levels (148).

Although recombinant *E. coli* XL1-Blue is able to synthesize substantial levels of P(3HB), growth is impaired by dramatic filamentation of the cells, especially in defined medium (143, 147, 285). By overexpression of FtsZ in this strain, biomass production was improved by 20% and P(3HB) levels were doubled (150). This recombinant strain produced 104 g of P(3HB) per liter in defined medium, corresponding to 70% of the cell dry weight. The volumetric productivity of 2 g/liter/h, however, is lower than achievable with *R. eutropha* (284).

One of the challenges of producing P(3HB) in recombinant organisms is the stable and constant expression of the *phb* genes during fermentation. P(3HB) production by recombinant organisms is often hampered by the loss of plasmid from the majority of the bacterial population. Such stability problems may be attributed to the metabolic load exerted by the need to replicate the plasmid and synthesize P(3HB), which diverts acetyl-CoA to P(3HB) rather than to biomass. In addition, plasmid copy numbers often decrease upon continued fermentation because only a few copies provide the required antibiotic resistance or prevent cell death by maintaining *parB*. For these reasons, Kidwell et al. designed a runaway plasmid to suppress the copy number of the plasmid at 30°C and induce plasmid replication by shifting the temperature to 38°C (119). By using this system, P(3HB) was produced to about 43% of the cell dry weight within 15 h after induction with a volumetric production of 1 g of P(3HB)/liter/h. Although this productivity is of the same order of magnitude as that of natural P(3HB) producers, strains harboring these *parB*-stabilized runaway replicons still lost the capacity to accumulate P(3HB) during prolonged fermentations.

Whereas the instability of the *phb* genes in high-cell-density fermentations affects the PHA cost by decreasing the cellular P(3HB) yields, another contributing factor to the compara-

tively high price of PHAs is the cost of the feedstock. The most common substrate used for P(3HB) production is glucose. Zhang et al. (303) examined *E. coli* and *Klebsiella aerogenes* strains for P(3HB) formation on molasses, which cost 33 to 50% less than glucose. The main carbon source in molasses is sucrose. Recombinant *E. coli* and *K. aerogenes* strains, carrying the *phb* locus on a plasmid, grown in minimal medium with 6% sugarcane molasses accumulated P(3HB) to approximately 3 g/liter, corresponding to 45% of the cell dry weight. When the *K. aerogenes* was grown in a fed-batch system in a 10-liter fermentor on molasses as the sole carbon source, P(3HB) was accumulated to 70% its cell dry weight, which corresponded to 24 g/liter. Although the *phb* plasmid in *K. aerogenes* was unstable, this strain shows promise as a P(3HB) producer on molasses, especially since *fadR* mutants incorporate 3HV up to 55% in the presence of propionate (303).

Morphologically, the number of granules in *E. coli* and *R. eutropha* and their size are not the same, even though they were synthesized by the same enzymes (170). By using differential scanning calorimetry, thermogravimetric analysis, and nuclear magnetic resonance, it was shown that the granules in *E. coli* are in a more crystalline form than the granules in *R. eutropha* (77). This may be because recombinant *E. coli* produces P(3HB) of higher molecular weight (133) or because of the absence of specific P(3HB)-binding proteins such as PhaP. The difference in crystallinity was thought to contribute to the differences in degradation of the polymer during purification (77). It was suggested that the increased crystallinity of this high-molecular-weight P(3HB) prevented the embrittlement seen for P(3HB) from natural sources such as *R. eutropha* (134), and recombinant P(3HB) may therefore have applications for which natural P(3HB) does not qualify.

As described above, the incorporation of other monomers in the growing P(3HB) chain results in polymers with drastically altered and improved mechanical properties. Therefore, recombinant production systems will have to be able to facilitate the production of a variety of copolymers.

P(3HB-3HV). Engineering *E. coli* to produce P(3HB-3HV) involved altering the endogenous metabolism of *E. coli* rather than introducing a specialized set of genes. Supplementation with propionate had generally been used for P(3HB-3HV) formation in *R. eutropha*, and the initial strategy for recombinant P(3HB-3HV) was therefore similar. Because *E. coli* does not readily import propionic acid, cultures were adapted on acetate and then a glucose-propionate mixture was added (243). This system was improved by using *E. coli* strains that have constitutive expression of the *ato* operon and *fad* regulon to fully express fatty acid utilization enzymes (54, 243). The *ato* system transports acetoacetate into the cell, and this is initially activated to acetoacetyl-CoA by AtoAD. AtoAD is also able to transport propionate into the cell (28) (Fig. 14). The *fad* regulon encodes enzymes for complete degradation of fatty acids, including a broad-specificity thiolase (28). It was expected that the FadA thiolase was beneficial in the pathway for 3HV formation compared to PhbA. The 3HV fraction in the copolymer was dependent on the percentage of propionate used during the fermentation, but it never exceeded 40%. Because *E. coli* is resistant to 100 mM propionate (243) whereas 30 mM is already toxic for *R. eutropha* (212), it was suggested that P(3HB-3HV) fermentations may be more efficient with *E. coli* strains (243).

In subsequent studies, propionyl-CoA formation was studied in strains with mutations in *ackA* and *pta* or in strains that overexpress Ack. For efficient incorporation of 3HV into P(3HB-3HV), *E. coli* requires the Pta and Ack activities (Fig. 14), although the acetate-inducible acetyl-CoA synthase may also be involved (227). The *prpE* product is a recently discovered acetyl-CoA synthase homolog which actually may be even more specific to propionate (94). The recombinant production systems for P(3HB-3HV) exemplify the need to alter the metabolism of *E. coli* as well as to adjust feeding strategies in order to produce the desired copolymers. As in *E. coli*, the *fadR* mutation also enables *Klebsiella oxytoca* to produce P(3HB-3HV) when grown on glucose and propionate (303).

Yim et al. reported that these recombinant *E. coli* P(3HB-3HV) producers are unable to grow to a high density and therefore are unsuited for commercial processes (301). In an attempt to improve P(3HB-3HV) production in a recombinant strain, four *E. coli* strains (XL1-Blue, JM109, HBJ01, and DH5α) were tested. All four recombinant *E. coli* strains synthesized P(3HB-3HV) when grown on glucose and propionate with HV fractions of 7% (301). Unlike the strains studied previously (243), recombinant XL1-Blue incorporated less than 10% HV when the propionic acid concentration was varied between 0 and 80 mM, HV incorporation and PHA formation were increased by pregrowing cells on acetate followed by glucose-propionate addition at a cell density of around 10^8 cells per ml. Oleate supplementation also stimulated HV incorporation. This recombinant XL1-Blue strain, when pregrown on acetate and with oleate supplementation, reached a cell density of 8 g/liter, 75% of which was P(3HB-3HV), with an HV fraction of 0.16 (301).

P(3HB-4HB) and P(4HB). P(4HB) is produced in *E. coli* by introducing genes from a metabolically unrelated pathway into a P(3HB) producer. The *hbcT* gene from *Clostridium kluyveri* encodes a 4-hydroxybutyric acid-CoA transferase (104). By engineering *hbcT* on the same plasmids as *phbC* from *R. eutropha*, recombinant *E. coli* produced 4HB-containing PHAs when grown in the presence of 4HB. Depending on the orientation of the *phbC* and *hbcT* genes in the vector and the growth conditions, up to 20% of the cell dry weight was made up of P(4HB) homopolymer. Interestingly, P(4HB) homopolymer was synthesized in the presence of glucose. In the absence of glucose, a P(3HB-4HB) copolymer was accumulated with up to 72% 3HB, even though *phbA* and *phbB* were absent. This suggests that *E. coli* contains an unknown pathway that allows the conversion of 4HB to 3HB (86).

Valentin and Dennis were able to produce P(3HB-4HB)

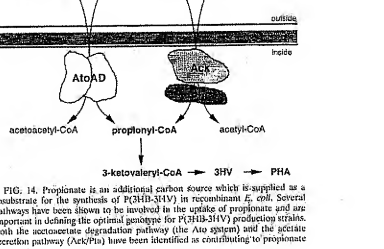

FIG. 14. Propionate is an additional carbon source which is supplied as a cosubstrate for the synthesis of P(3HB-3HV) in recombinant *E. coli*. Several pathways have been shown to be involved in the uptake of propionate and are important in defining the optimal genotype for P(3HB-3HV) production strains. Both the acetoacetate degradation pathway (the Ato system) and the acetate secretion pathway (Ack/Pta) have been identified as contributing to propionate transport.

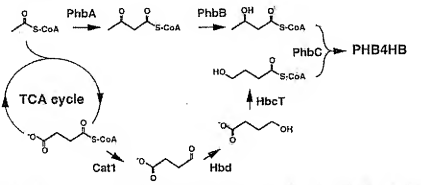

FIG. 15. Biosynthesis of P(3HB-4HB) in recombinant *E. coli* by using heterologous genes from *Clostridium kluyveri*. The 4HB monomer in the synthesis of P(3HB-4HB) is derived from succinate. Succinate is converted to 4HB-CoA by enzymes that are encoded by genes from the gram-positive, strictly anaerobic *C. kluyveri* microbe.

directly from glucose (276). This was accomplished by introducing the succinate degradation pathway from *C. kluyveri* on a separate plasmid into an *E. coli* strain harboring a plasmid with the *phb* biosynthetic genes from *R. eutropha*. This copolymer is synthesized by redirecting succinyl-CoA from the TCA cycle to 4-hydroxybutyryl-CoA via succinic semialdehyde and 4HB (Fig. 15). P(3HB-4HB) was accumulated to 46% of the cell dry weight with a 1.5% 4HB (276).

P(3HO-3HH). *E. coli* has also been engineered to produce msc-PHAs by introducing the *phaC1* and *phaC2* gene from *P. aeruginosa* in a *fadB*::Kan mutant (136, 211). It was presumed that this mutant accumulated intermediates of the β-oxidation pathway that could be incorporated into PHA by the polymerases. The recombinant *E. coli* strain accumulated PHA up to 21% of the cell dry weight when grown in LB broth containing decanoate. The polymer contained primarily 3-hydroxydecanoate (73%) and 3-hydroxyoctanoate (19.0%) (136). Interestingly, the *fadB* mutation in this strain is an insertion mutation and not a point mutation and is noted to have undetectable FadAB activity. If FadAB is the only β-oxidation complex in *E. coli*, one would expect that this strain would not be capable of degrading fatty acids to PHA monomer.

The *phaC1* gene from *P. oleovorans* also directs PHA formation in *E. coli*. Strains with a *fadA* or *fadB* mutation accumulated PHA up to 12% of the cell dry weight when grown on C_8 to C_{12} fatty acids. By replacing the wild-type promoter of *phaC1* with either the *alk* or *lac* promoter, polymerase levels were inducible, leading to 20 to 30% PHA formation with PHA polymerase 1 or 2. These experiments show that PHA polymerase is the only dedicated enzyme for PHA biosynthesis in *Pseudomonas* and that additional enzyme activities may be provided by ancillary enzymes (217).

Conclusions

With the identification of *pha* genes from multiple organisms, the possibilities of constructing recombinant PHA producers have emerged. History has repeated itself in that P3HB was again the first biological polyester, but now from a recombinant microorganism. The diversity of natural PHAs, however, was rapidly conferred to *E. coli*, and several ssc-PHAs and msc-PHAs have been synthesized in recombinant bacteria, albeit with various degrees of success. Significant progress must be made to produce a variety of PHAs in recombinant bacteria by cofeeding strategies, let alone from single-carbon sources. The optimization of fermentation systems for these recombinant organisms will also remain a challenge. Since PHAs are not natural products of *E. coli*, the responses by high-cell-density cultures to nutrient limitations that trigger subsequent feeds are unpredictable. New fermentation feeding strategies will therefore have to be developed.

METABOLIC ENGINEERING OF PHA BIOSYNTHETIC PATHWAYS IN HIGHER ORGANISMS

In an effort to reduce the cost of P(3HB) production, industrial interest has initiated programs for P(3HB) production systems in plant crops. Commercial oil-producing crops, such as *Brassica*, sunflower, or corn, have been bred to accumulate these oils to high levels. If one were able to replace the oil by PHAs and have the polymer be accumulated to 30% of the seed, PHA production per acre could be around 350 lb. Production of 1 billion lb of PHA would then require an area of 2.5 million acres (8% of the state of Iowa). The potential of an agricultural PHA production system is thus enormous (293). The prospects of producing P(3HB) in plant crops is encouraging now that several studies have reported the synthesis of PHAs in yeast, insect cells, and several plant species.

Saccharomyces cerevisiae

In contrast to *E. coli*, where the complete P(3HB) pathway had to be introduced for PHA formation to occur, P(3HB) was produced in yeast by expressing only part of the biosynthetic pathway. P(3HB) granules could be visualized in *Saccharomyces cerevisiae* cells when just the P(3HB) polymerase gene from *R. eutropha* was introduced into the cells. However, P(3HB) was accumulated to only 0.5% of the cell dry weight. This low level of P(3HB) may result from insufficient activity of the endogenous β-ketoacyl-CoA thiolase and acetoacetyl-CoA reductase enzymes. β-Ketoacyl-CoA thiolase (10 to 20 nmol/min/mg) and acetoacetyl-CoA reductase (150 to 200 nmol/min/mg) were detected and were thought to supply sufficient substrate for P(3HB) polymerase (138). Future improvements of this eukaryotic P(3HB) production system may require elevation of these activities.

Insect Cells

Expression of the *R. eutropha phbC* gene in insect cells was first achieved in *Trichoplusia ni* (cabbage looper) cells by using a baculovirus system. Expression of *phbC* was successful, since within 60 h after viral infection, 50% of the total protein was P(3HB) polymerase. In contrast to other recombinant systems, expression of *phbC* in insect cells allowed rapid purification of

the soluble form of P(3HB) polymerase (291). This is surprising, since overexpression of PhbC in recombinant *E. coli* usually results in insoluble, inactive P(3HB) polymerase.

An elegant study with insect cells attempted to create a diverse set of PHA monomers endogenously by transfecting a mutant form of the rat fatty acid synthase into *Spodoptera frugiperda* (fall armyworm) cells by using a baculovirus (292). This previously characterized fatty acid synthase mutant does not extend fatty acids beyond 3HB (113), which was subsequently converted to P(3HB) by the cotransfected P(3HB) polymerase from *R. eutropha*. The presence of P(3HB) granules in the insect cells was visualized by immunofluorescence. Although P(3HB) production was achieved, only 1 mg of P(3HB) was isolated from 1 liter of cells, corresponding to 0.16% of the cell dry weight. These studies provide examples of the use of alternative, eukaryotic enzymes for the generation of P(3HB) intermediates and the ability to express the *phb* genes in heterologous hosts (292).

Plants

Recently, efforts have been made to produce P(3HB) in plants. Stable expression of the *phb* genes has been achieved and the P(3HB) produced is chemically identical to the bacterial products with respect to the thermal properties (T_m, T_g, ΔH), while the molecular weight distribution of the polymer was much broader. Still, a significant fraction of the plant P(3HB) had a molecular weight of 1,000,000, which indicated that plants can make P(3HB) of sufficient quality for industrial processing (200).

Since, in contrast to bacteria, eukaryotic cells are highly compartmentalized, there are a number of challenges in expressing *phb* genes in plants. *phb* genes must be targeted to the compartment of the plant cells where the concentration of acetyl-CoA is the highest but only in such a way that growth of the plant is not restricted.

Arabidopsis thaliana. Although not a crop plant, *Arabidopsis thaliana* was the first plant of choice for transgenic P(3HB) studies since it is the model organism for heterologous expression studies in plants. The only enzyme of the P(3HB) synthesis pathway naturally found in *A. thaliana* is 3-ketoacyl-CoA thiolase. This cytoplasmic 3-ketoacyl-CoA thiolase produces mevalonate, the precursor of isoprenoids. Because of the presence of endogenous thiolase activity, only the *phbB* and *phbC* genes from *R. eutropha* were transfected, resulting in the accumulation of P(3HB) granules in the cytoplasm, vacuole, and nucleus. The expression of the *phb* genes had an adverse effect on growth which was possibly due to the depletion of acetyl-CoA from an essential biosynthetic pathway. Alternatively, P(3HB) accumulation in the nucleus could be detrimental (199). Similar growth defects and low P(3HB) yield were obtained with the commercial crop *Brassica napus*. These problems could not be surmounted by introducing *phbA* in the presence of *phbB* and *phbC*. This suggests that the endogenous thiolase activity may not have been the critical factor in the phenotypic problems associated to P(3HB) synthesis (178).

An improved plant production system was subsequently developed by expressing all three *phb* genes in the plastid of *A. thaliana*. The plastid was targeted for P(3HB) production because of the high level of acetyl-CoA in this organelle, which is the site for lipid biosynthesis. The P(3HB) content in the plastids gradually increased over time, and the maximum amount of P(3HB) in the leaves was 14% of the dry weight (179). In contrast to the broad molecular mass distribution of P(3HB) produced in the cytoplasm (200), P(3HB) isolated from the plastids had a uniform molecular mass of 500,000 Da (177).

Gossypium hirsutum (cotton). Recently *phb* genes were engineered into cotton (*Gossypium hirsutum*) to determine whether P(3HB) formation could alter the characteristics of the cotton fiber. Constructs containing *phbB* and *phbC* were targeted to fiber cells. Expression of these constructs was switched on in the early fiber development stages (10 to 15 days postanthesis), under the control of the E6 promoter, or during the late fiber development stages (35 to 40 days postanthesis), when the genes were under the control of the FbL2A promoter. In the fibers of the transgenic plants, the endogenous thiolase activity varied between 0.01 and 0.03 μmol/min/mg and the reductase activity varied between 0.07 and 0.52 μmol/min/mg. Epifluorescence studies showed that P(3HB) granules had been deposited in the cytoplasm (112). Due to the presence of P(3HB) granules in the cotton fiber, the heat capacity of the purified cotton was increased and better insulation properties were obtained (26). Further improvement of P(3HB) and cotton fiber compositions is expected to improve cotton characteristics with respect to dyeability, warmth, and wrinklibility. Even though the maximum levels of P(3HB) amounted to only 3.4 mg/g of dry fiber, the incorporation of P(3HB) to this level already showed an effect.

Zea mays (corn). The P(3HB) biosynthetic pathway from *R. eutropha* has also been expressed in Black Mexican sweet maize (*Zea mays* L.) cell cultures. Cell cultures were grown in a bioreactor for 2 years rather than in fully differentiated plants. The thiolase activity (0.140 U/mg) was constant, but the reductase activity was less stable and decreased from 0.64 to 0.12 U/mg. The *phbC* gene was initially detected, but after 1.5 years of cultivation it had been lost. In addition to the instability of the *phbB* and *phbC* genes, the transformed plant cells grew more slowly than the native cells did (75).

Conclusions

Although P(3HB) synthesis has been achieved in plants, the results obtained so far clearly indicate that a long road is still ahead. In contrast to microorganisms, metabolism in plants is mostly compartmentalized, which complicates the tasks at hand. Current and future developments in the molecular biology of plants will undoubtedly find rapid application in the pursuit of PHAs in plant crops. An intriguing development is the potential for transgenic P(3HB) to play a role in engineering new characteristics into existing materials such as cotton. Obviously, the limits of transgenic PHA production are unpredictable.

POTENTIAL ROLE FOR PHAs IN NATURE

Since bacteria did not evolve PHA production as a means of supplying plastics to mankind, the accumulation of PHAs by bacteria must have evolved out of an advantageous phenotype related to the deposition of these materials. Besides the discussed role as storage material for carbon and reducing equivalents, low-molecular-weight P(3HB) has been found to be part of bacterial Ca^{2+} channels and is also bound to protein and lipids in eukaryotic systems.

Voltage-Dependent Calcium Channel in *Escherichia coli*

An extensive body of knowledge was developed by Rosetta Reusch and coworkers at Michigan State University on the possible role and function of low-molecular-weight P(3HB) in microbial physiology (98, 99, 219, 223, 224). Recently it was established that P(3HB) in conjunction with polyphosphate can form a complex in *E. coli* that transports calcium ions. A

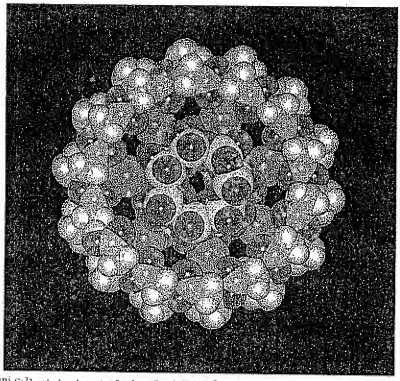

FIG. 16. Model of the P(3HB) Ca^{2+}-polyphosphate complex from *E. coli*. This P(3HB) complex forms a channel in the membrane to transport Ca^{2+} ions out of the cell. It is proposed that the channel is also involved in DNA uptake by competent *E. coli* cells. In this model, the Ca^{2+} ions (green) are localized between the inner polyphosphate molecule (yellow phosphorus atoms and red oxygen atoms) and a P(3HB) helix (red oxygen atoms, blue carbon atoms, and white hydrogen atoms). The methyl side groups of the P(3HB) helix face the outside of the channel and are in contact with the hydrophobic lipids of the membrane. The carbonyl oxygen atoms face the interior of the channel and ligand the Ca^{2+} ions. The phosphate groups play a similar role. Extrusion of Ca^{2+} ions may result from physical constraints on the structure or from enzymatic synthesis and degradation of the polyphosphate chain at the membrane/cytosol and membrane/periplasm interfaces.

model of such a complex is shown in Fig. 16. An alternative model has been based on the crystal structure of pure P(3HB) oligomers; however, that structure does not take the polyphosphate molecule into account (238).

Complexed P(3HB) (cPHB) is a low-molecular-mass P(3HB) (less than 15,000 Da) that has been found in low concentrations attached to cellular proteins (99) or complexed with calcium and polyphosphate in the form of a calcium channel in the cytoplasmic membrane (219, 224). It has been proposed that these latter structures aid the import of DNA after cells have been made genetically competent in procedures that use calcium ions. When cultures of *A. vinelandii*, *Bacillus subtilis*, *Haemophilus influenzae*, and *E. coli* are treated to make them genetically competent for DNA uptake, a specific change in the structure of the membrane of these cells is detected by fluorescence studies (223). Comparative studies indicated a close relationship between genetic competence, the appearance of this characteristic change in membrane structure, and the cPHB content of *E. coli* cells. In these studies, the transformation buffer that is generally used to make *E. coli* cells competent was varied such that instead of Ca^{2+} ions, a broad range of mono-, di-, and trivalent cations were examined for their capacity to make cells prone to take up DNA. From these studies, it was clear that only Ca^{2+} and Mg^{2+} ions can establish the competence state and that some ions support low efficiencies of transformation or even inhibit DNA uptake completely. For each metal ion, the transformation efficiency was closely related to the structure of the membrane as observed by fluorescence studies (98).

Because this type of P(3HB) is so different from the P(3HB) in the storage granules, new assays were developed to determine the amount of P(3HB) in biological samples. By using these techniques, it has been shown that competent *E. coli* cells contain cPHB in their cytoplasmic membranes and that the presence of cPHB was directly related to the transformability of the cells. The molar ratio of the components of the P(3HB)-polyphosphate-Ca^{2+} complex was determined from cPHB purified from genetically competent *E. coli* to be 1:1:0.5. These isolated cPHB complexes were able to form Ca^{2+} channels when introduced into liposomes (224) or voltage-activated Ca^{2+} channels in lipid bilayers. Identification of this channel as a calcium channel constitutes the first known biological nonproteinaceous Ca^{2+} channel (219). At present, no information is available for the genes and the corresponding gene products that are participating in cPHB biosynthesis. The elucidated genomic sequence of *E. coli* (12) does not show any significant homolog of a PHA polymerase-encoding gene.

Subsequent work proved that a channel with identical properties can be reconstituted from Ca^{2+} polyphosphate and synthetically prepared (*R*)-3-hydroxybutyrate oligomers (33). Recently, P(3HB) and polyphosphate have also been identified as components of purified Ca^{2+}-ATPase from the human erythrocyte, a well-studied Ca^{2+} channel (220). Given the relative simplicity of the P(3HB)-polyphosphate complex in comparison with the proteinaceous Ca^{2+} channels, it is tempting to consider the possibility that these bacterial channels have a primordial origin.

Low-Molecular-Weight PHB in Eukaryotic Organisms

P(3HB) is not just an insoluble molecule made by bacteria but, rather, is a unique compound with a variety of roles and functions in nature. P(3HB) has also been found in a variety of plant and animal tissues (218). In human plasma, P(3HB) can be found associated with very-low-density lipoprotein and low-density lipoprotein, but not with high-density lipoprotein. In addition, a significant portion of P(3HB) is found associated with serum albumin. The lipid molecules and albumin are thought to be acting as transporters of P(3HB) through the blood, with albumin being the major carrier (225). If P(3HB) plays a physiological role in large eukaryotic organisms, the need for a P(3HB) carrier makes sense, since P(3HB) is highly insoluble in aqueous solutions.

Possible Evolutionary Precursors of PHB

Since PHB is such a high-molecular-weight molecule, it becomes an intriguing question to find which cellular function has driven its evolution. The direct involvement of DNA, RNA, and protein in sustaining life provides a simple clue for the presence of these macromolecules in the living cell. PHA, however, seems to be an inert molecule, and, as with polysaccharides, it is interesting to speculate about the roots of such molecules. Intracellular stores are obviously advantageous during prolonged periods of starvation, but what was the evolutionary, low-molecular-weight precursor? Why were 3-hydroxyacyl-CoAs found to be good substrates for deposition in intracellular granules, and could they have been abundant in the cell during starvation? Where did the enzymes that facilitate PHA synthesis come from? The most obvious hypothesis for its original biosynthetic pathway is suggested by similarities of its monomers to intermediates of fatty acid metabolism. 3-Hydroxy fatty acids are part of fatty acid biosynthesis and degradation, and these pathways do involve a β-ketoacyl-CoA thiolase and β-ketoacyl dehydrogenase. However, PHA polymerase, the enzyme involved in the unique step in PHA biosynthesis, does not have any significant homology to other proteins, and its evolutionary predecessor remains enigmatic.

By analogy, one can speculate about the origin of other ubiquitous storage materials such as starch, glycogen, or natural rubber. For these polymers, an evolutionary predecessor should also have a more essential function than being a storage molecule. Several oligosaccharides are essential for a bacterium. Trehalose is a dimer of glucose molecules and serves as an osmoprotectant for the cell. Lipopolysaccharides are oligosaccharides linked to diacylglycerol moieties and play a role in maintaining cell integrity and viability. Limited polymerization of glucose may have been an early evolutionary step in the eventual pathway to polysaccharides such as glycogen and starch. Other polysaccharides may have been synthesized by analogous pathways built on this scheme. In that context, oligomers of P(3HB) may have been, or may still be, important for life. Recently, oligomers of (*R*)-3-hydroxybutyrate were identified as pheromones in spiders (237). The P(3HB) component of Ca^{2+} channels and perhaps other transporters may be a subsequent low-molecular-weight predecessor of the high-molecular-weight material. Although unrelated to commercial PHA production, this evolutionary perspective suggests that cPHB may become a new paradigm in microbial physiology or even biology in general. As such, it may provide additional and unexpected clues to the future of biological polyesters.

CONCLUSIONS

An immense body of information is available presently to engineer organisms for the synthesis of almost any PHA. A polymerase-encoding gene for a specific composition can be chosen from a set of 18 identified genes. Depending on the pathway to be used for generating the desired monomers, *phbAB*, *phaI*, or *phaG* genes are available. These can be chosen from a number of different organisms as well. In addition to these essential *phb* genes, other enzymes may be used to generate novel monomers. The opportunities seem limitless.

Recombinant production of molecules such as PHAs will undoubtedly thrive on the enormous biological diversity of nature, where novel protein activities can be obtained from exotic places, while gene cloning becomes less and less of a technological hurdle. In the future, bacterial fermentations will be able to support the production of a wide range of PHAs. For economic reasons, plant crops promise to be a more desired vehicle for PHA production. New procedures to introduce and express genes in plants are generated rapidly and will enable the timely expression of desired genes in the compartments of choice. Enzymes with all the desired characteristics will furthermore be obtained by new in vitro molecular breeding approaches as long as the screening tools are available. It is clear that at the start of the third millennium, transgenic PHA producers will be an important source of green plastics and chemicals to the world. With the advent of further developments in metabolic engineering, such biotechnologies will be the rule rather than the exception.

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